DATA EVALUATION RECORD

DEMIDITRAZ
[PF-03814927]

STUDY TYPE: DEVELOPMENTAL NEUROTOXICITY STUDY - RAT;
OCSPP 870.6300 (§83 6); OECD 426 (DRAFT)

MRIDS 48766703 and 48766701

Prepared for

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DATA EVALUATION RECORD

STUDY TYPE: Developmental Neurotoxicity Study - Rat;
OCSPP 870.6300 (§83-6); OECD 426 (draft)

PC CODE: 577501 **DP BARCODE:** D400484

TEST MATERIAL (PURITY): Demiditraz (100% a.i.)

SYNONYMS: PF-03814927

CITATION: Beck, M.J. (2012) An oral (gavage) developmental neurotoxicity study of Demiditraz (PF-03814927) in rats. WIL Research Laboraties, LLC, Ashland, Ohio. Laboratory Project ID WIL-344066, Study Initiation April 4, 2011 - Study Completion February 1, 2012. MRID 48766703. Unpublished.

Beck, M.J. (2011) An oral (gavage) preliminary developmental neurotoxicity (DNT) study of Demiditraz (PF-03814927) in rats, including exposure assessment. WIL Research Laboratories, LLC, Ashland, Ohio. Laboratory Project ID WIL-344067, Study Initiation March 8, 2011 - Study Completion January 20, 2012. MRID 48766701. Unpublished.

SPONSOR: Pfizer, Inc., 7000 Portage Road, Kalamazoo, MI 49001

EXECUTIVE SUMMARY:

In a developmental neurotoxicity study (MRID 48766703) Demiditraz (PF-03814927) (100% purity; Batch 1 WIL ID No. 110036/Lot # TCK08005K and Batch 2 – WIL ID No. 110040/Lot # TCK08005K) was administered daily by oral gavage to 25 mated female Sprague-Dawley [Crl:CD(SD)] rats per dose at dose levels of 0 (vehicle), 5, 15, or 100 mg/kg bw/day from gestation day (GD) 6 through lactation day (LD) 20. The vehicle used in preparation of the test substance formulations and for administration to the control group was 0.5% methylcellulose (400 cps) and 0.1% Tween® 80 in deionized water. The experimental females were approximately 14 weeks of age at the beginning of test substance administration. Dose selection was based on a preliminary study (MRID 48766701), which also provided verification of exposure to pups.

Procedurally, all animals were observed twice daily for appearance and behavior. Clinical observations, body weights, and food consumption were recorded at appropriate intervals. In addition, detailed clinical observations were conducted on all dams on GDs 10 and 15 and on LDs 10 and 20. All females were allowed to deliver and rear their offspring to LD 21. All surviving F_0 females were euthanized on LD 21 and subjected to a gross necropsy. Clinical observations, body weights, and sexes were recorded for the F_1 pups at appropriate intervals. On

postnatal day (PND) 4, litters were culled to 8 pups/litter. Following culling, a subset (Subset A) of 20 pups/sex/group was assigned to detailed clinical observations (i.e., functional observational battery or FOB) (PND 4, 11, 21, 35, 45, and 60), auditory startle response (PND 20 and 60), locomotor activity (PND 13, 17, 21, and 61), and learning and memory (PND 62). From this subset, 15 pups/sex/group were deeply anesthetized and perfused in situ on PND 72 for brain weight and measurement evaluations; of these, 10 pups/sex/group were selected for neuropathological and brain morphometric evaluations on PND 72. A second subset (Subset B) of 20 pups/sex/group was selected for learning and memory (PND 22). A third subset (Subset C) of 15 pups/sex/group was deeply anesthetized and perfused in situ on PND 21 for brain weight and measurement evaluations; of these, 10 pups/sex/group were selected for neuropathological and brain morphometric evaluations on PND 21. Indicators of physical development (balanopreputial separation and vaginal patency) were evaluated for all F_1 selected animals in Subsets A and B. All F_1 animals not selected for neuropathological or behavioral evaluations were euthanized and necropsied on PND 21. F_1 animals selected for learning and memory assessment on PND 22 were necropsied following attainment of sexual developmental landmarks. The litter was used as the experimental unit for all F_1 data.

One F_0 female in the 100 mg/kg/day group was found dead on GD 21, and cause of death could not be determined. All other F_0 females survived to the scheduled euthanasia. One female each in the control and 100 mg/kg/day groups failed to deliver and were determined to be nongravid. Test substance-related clinical findings were observed in a dose-dependent manner for the 15 and 100 mg/kg/day group F_0 females and were observed at approximately 15-30 minutes post-dosing throughout the treatment period. In both dose groups these findings included sitting with the head held low, hypoactivity, a flattened body, slightly drooping eyelids, decreased respiration, clear material around the mouth/salivation, lacrimation, and/or dilated pupils, and additional signs seen only in the 100 mg/kg/day dose group included rocking, lurching, or swaying while ambulating, piloerection, tremors/convulsions, and yellow material on various body surfaces. These findings generally continued to the 2-hour post-dosing observations for the 100 mg/kg/day group F_0 females, but at decreased incidences, and were not observed the following morning at the daily clinical observations prior to dose administration. There were no test substance-related clinical findings observed for F_0 females in the 5 mg/kg/day group.

Test substance-related decrements in maternal care were observed in the 100 mg/kg/day group F_0 females at approximately 15-30 minutes post-dosing and continued, although somewhat abated, to the 2-hour post-dosing observations. A decreased incidence of F_0 females on the nest, a higher incidence of females that were away from the nest (not actively eating, drinking, grooming or tending to the litter), and higher incidences of scattered litters with 1-3 pups and more than 3 pups outside of the nest were all noted at the 100 mg/kg/day dose level. The 5 and 15 mg/kg/day groups of F_0 females also had slightly increased incidences of females away from the nest (but not actively eating, drinking, grooming or tending to the litter) and incidences of scattered litters with 1-3 pups outside the nest at 15-30 minutes post-dosing; the slightly increased incidences of F_0 females being away from the nest appeared to continue into the 2-hour post dose period for both of these groups, but the slightly increased incidence of scattered litters continued only for the 15 mg/kg/day dose group. In the absence of any other treatment-related effects in the 5 mg/kg/day group of F_0 females, the apparent slight changes maternal behavior at this dose level are not considered biologically relevant.

During the detailed clinical observations (FOB) for F_0 females on GDs 10 and 15 and LDs 10 and 20, a flattened posture or sitting with the head held low, low or very low arousal, slight to

moderately coarse tremors, lacrimation, salivation, piloerection, slightly drooping eyelids, decreased respiration, soft and flabby muscle tone, impaired mobility, ataxia, dragging bodies, and/or higher urination counts were observed for the 100 mg/kg/day group. At 15 mg/kg/day, sitting with the head held low, a flattened posture, ataxia, impaired mobility, and a higher urination count were observed in a smaller number of F_0 females beginning on GD 15.

F_0 females in the 100 mg/kg/day group had slightly lower mean body weight gains throughout the gestation treatment period (cumulative during GDs 6-20, 15% less than controls; $p<0.01$), and corresponding reductions in food consumption were noted during GDs 6-9 and 9-12 (13-14% less than controls; $p<0.01$). As a result, mean body weights for F_0 females in the 100 mg/kg/day group were 4.6% lower than the control group by GD 20 ($p<0.05$). A lower mean body weight gain was noted for F_0 females in the 100 mg/kg/day group during LDs 1-4 (67% less than controls; $p<0.01$), but mean body weight gains for this group were generally similar to the control group for the remainder of the lactation treatment period (LDs 4-21), despite lower food consumption in this group generally throughout the lactation period (10-16% less than controls; $p<0.01$). Mean body weights for F_0 females in the 100 mg/kg/day group were 4.5% to 6.3% lower than the control group during LDs 4-17, but were similar to the control group on LD 21. Mean gestation length of F_0 females treated at 100 mg/kg/day was slightly, but statistically and biologically significantly increased (22.2 days vs. 21.8 days for controls). There were no treatment-related effects on the process of parturition or macroscopic findings of the F_0 females.

The maternal LOAEL for Demiditraz administered to pregnant Sprague-Dawley rats by oral gavage once daily from GD 6 to LD 20 is 15 mg/kg bw/day, based on adverse clinical findings (sitting with the head held low, hypoactivity, a flattened body, slightly drooping eyelids, decreased respiration, clear material around the mouth/salivation, lacrimation, and/or dilated pupils) and slightly increased post-dosing incidences of findings indicative of diminished maternal care (dam away from the nest, but not eating, drinking, grooming, or tending to the litter, and 1-3 pups outside of the nest). The corresponding maternal NOAEL is 5 mg/kg/day.

The mean live litter size in the 100 mg/kg/day group was slightly lower than the control group (14.0 vs. 15.1 for controls), and mean postnatal survival for the 100 mg/kg/day group was lower than the control group during PND 0-1, 1-4 (pre-culling), and birth to PND 4 (pre-culling) (87.7% vs. 96.2% for the latter). Among the clinical findings, it was noted that at the 100 mg/kg/day dose level a total of 24 pups were found dead and 20 others were missing (presumed cannibalized) compared with 11 found dead and 3 missing at the control level. At 100 mg/kg/day, F_1 pups of both sexes had decreased body weights and body weight gains generally throughout the pre-weaning period, relative to their respective controls.

During the post-weaning interval, test substance-related, lower mean body weight gains were noted for the 100 mg/kg/day group F_1 males when compared with the control group during PND 28-56 and when the overall post-weaning period was evaluated (PND 28-72). As a result, mean body weights for the 100 mg/kg/day group F_1 males were significantly lower than the control group during PND 28-72. A test substance-related lower mean body weight gain was noted for the 100 mg/kg/day group F_1 females during PND 28-35, but mean body weight gains for these females were similar to the control group for the remainder of the post-weaning period and when the entire post-weaning period (PND 28-72) was evaluated. Mean body weights for the 100 mg/kg/day group F_1 females were lower than the control group during PND 28-72. Mean post-weaning offspring body weights and body weight gains in the 5 and 15 mg/kg/day groups were

unaffected by treatment.

A test substance-related delay in the mean age of attainment of balanopreputial separation was noted for the 100 mg/kg/day group F_1 males (47.0 days vs. 44.3 days for controls) but mean body weights of the F_1 males on the day of attainment were not affected at any dose level. In contrast the F_1 females showed no treatment related effects on the mean age of attainment of vaginal patency, but a reduced mean body weight on the day of attainment of vaginal patency was noted for the F_1 females of the 100 mg/kg/day group.

Mean forelimb grip strength for the 100 mg/kg/day group F_1 males and females was significantly lower than the control group on PND 21 and 3 but was no longer significantly affected on PND 45 or PND 60. There were no effects on grip strength at the 5 and 15 mg/kg/day dose levels on any day of evaluation.

There were no significant effects on locomotor activity (mean total and ambulatory counts) or habituation within each test session in either male or female F_1 offspring that could be attributable to demiditraz at any dose level on PND 13, 17, 21, and 61. The investigator stated that there was a suggestion of a non-significant treatment-related effect on the pattern of activity across the PND 13-21 period in males at the 100 mg/kg/day dose level and considered this consistent with a generalized delay in development at this dose level.

On PND 60, the mean maximum startle response (MAX) for the 100 mg/kg/day group (combined sexes) was higher than the control group throughout the testing session. There was no effect on MAX on PND 20 at all dose levels or PND 60 at 5 and 15 mg/kg/day. In addition, no effects were noted in the pattern of the habituation response over the entire 50-block test session in adult animals.

At 100 mg/kg/day, mean brain weight relative to final body weight was increased in F_1 pups of both sexes on PND 21 and in males, only, on PND 72, relative to corresponding controls, indicating a relative sparing of the brain weight despite the lower body weights for this dose group during the pre-weaning period. Brain weights were not affected at the 5 or 15 mg/kg/day dose levels.

There were no test substance-related effects on the numbers of former implantation sites or unaccounted-for sites, the pup sex ratio at birth, pup survival following culling, clinical findings at the weekly examinations, swimming ability or learning and memory assessments, macroscopic internal findings, gross or microscopic observations of the brain or peripheral nervous systems, brain measurements at necropsy, or morphometry taken from brain sections. The detailed clinical observations on PND 4, 11, 21, 35, 45, or 60 revealed no treatment related effects on home cage, handling, open field, or sensory observations.

The F_1 offspring LOAEL for Demiditraz administered to pregnant Sprague-Dawley rats by oral gavage once daily from GD 6 to LD 20 is 100 mg/kg/day, based on decreased postnatal survival, decreased pup body weights and body weight gains, increased maximum response to the auditory startle stimulus, decreased total and ambulatory motor activity counts (across, but not within sessions), decreased grip strength, and delayed attainment of balanopreputial separation. The corresponding F_1 offspring NOAEL is 15 mg/kg/day.

This study is classified **Acceptable/Guideline** and **satisfies** the guideline requirement for a

developmental neurotoxicity study in rats [OCSPP 870.6300 (§83-6); OECD 426 (draft)].

COMPLIANCE: Signed and dated GLP Compliance, Quality Assurance Approval, and No Claim of Confidentiality statements were provided. No deviations from regulatory requirements are noted.

I. MATERIALS AND METHODS:

A. MATERIALS:

1.	**Test material:**	Demiditraz (PF-03814927)
	Description:	White, crystalline powder, received from Sponsor (Pfizer, Inc.)
	Lot/batch #:	Lot No. TCK08005K/ Batch 1 WIL ID #110036 received 2/22/11 exp. Date 4/12/12 and Batch 2 WIL ID #110040 received 3/2/11 exp. Date 4/12/12
	Purity:	100 % a.i.
	Compound stability:	Stable under room temperature
	CAS # of TGAI:	Not available
	Structure:	Not available

2. Vehicle and/or positive control: The vehicle was 0.5% methylcellulose (400 cps) and 0.1% Tween 80 in deionized water:

- Methylcellulose, 400 cps (lot no. 060M0123V, exp. Date: 1 May 2013, received from Sigma-Aldrich Corporation, St. Louis, MO)
- Polysorbate 80, NF (lot no. ZM0688, exp. Date: 30 November 2011, received from Spectrum Chemical Manufacturing Corporation, New Brunswick, NJ)
- De-ionized water prepared on-site

3. Test animals (P):

Species:	Rat
Strain:	Sprague Dawley [Crl:CD(SD)]
Age at study initiation:	Approximately 13 wks at mating
Wt. at study initiation:	227 - 297 g on gestation day 0
Source:	Charles River Laboratories, Inc., Raleigh, NC
Housing:	Upon arrival and until pairing, all animals were individually housed in clean, stainless steel wire-mesh cages suspended above cage-board. The animals were paired for mating in the home cage of the resident male. Following positive evidence of mating, the females were individually housed in plastic maternity cages with nesting material, ground corncob bedding (Bed-O'Cobs®;The Andersons, Cob Products Division, Maumee, OH). The females were housed in these cages through lactation day 21, the scheduled day of necropsy.
Diet:	PMI Nutrition International, LLC Certified Rodent LabDiet® 5002 (a certified feed with appropriate analyses performed by the manufacturer and provided to WIL Research), *ad libitum*
Water:	On-site reverse-osmosis-purified drinking water, delivered by an automatic watering system, *ad libitum*
Environmental conditions:	Temperature: 22°C ± 3°C Humidity: 50% ± 20% Air changes: 10/hr Photoperiod: 12 hrs dark/ 12 hrs light (0600 hrs to 1800 hrs)
Acclimation period:	A minimum of 20 days

B. PROCEDURES AND STUDY DESIGN:

1. **In life dates:** Start: April 5, 2011; End: July 27, 2011.

2. **Study schedule:** After acclimation, the sexually mature virgin female rats were mated and assigned to four treatment groups. The test substance (Demiditraz) or vehicle was administered to the maternal animals daily by oral gavage from gestation day (GD) 6 through postnatal day (PND) 20, with GD 0 defined as the day on which mating was confirmed, and PND 0 defined as the day on which parturition was initiated. Litter standardization (culling) and selection of F_1 pups were conducted on PND 4. Pups were weaned on PND 21, after which time all surviving F_0 female animals were euthanized. Testing of F_1 pups continued until PND 72 (study termination).

3. **Mating procedure:** After acclimation, each female animal was placed in a suspended wire-mesh cage with a resident male from the same strain and source for mating. Each mating pair was examined daily. Positive evidence of mating was confirmed by the presence of a vaginal copulatory plug or the presence of sperm in a vaginal lavage and verified by a second biologist. The day on which evidence of mating was obtained was considered gestation day 0. Following positive evidence of mating, the females were individually housed in plastic maternity cages with nesting material, ground corn-cob bedding. The females were housed in these cages through lactation day 21, the scheduled day of necropsy.

4. **Animal assignment:** Mated females were assigned, using an in-house computer program which randomized the animals based on stratification of the gestation day 0 body weights in a block design, to one of four treatment groups as indicated in Table 1. Dams were assigned to functional observation testing as shown.

   Following litter standardization (i.e., culling) on postnatal day 4, offspring were assigned to one of three testing Subsets (A, B, C). To form each Subset, 1 pup/sex/litter was randomly assigned until the desired number of pups/sex/group for that Subset was attained. The group size (N) for each Subset and the assigned experimental parameters for which each Subset of offspring was used are described below and presented in Table 1.

   Subset A of 20 pups/sex/group was assigned to detailed clinical observations (PND 4, 11, 21, 35, 45, and 60), auditory startle response (PND 20 and 60), locomotor activity (PND 13, 17, 21, and 61), and learning and memory (PND 62). Note that the all pups were used for each of these experimental parameters. Indicators of physical development (balanopreputial separation and vaginal patency) were also evaluated for all F_1 selected animals in Subset A. Following completion of all behavioral testing, 15 animals/sex/group from Subset A (1 male and/or 1 female from each of the litters represented in Subset A) were selected for brain weight and measurement evaluations on PND 72; the animals were selected using a systematic approach to ensure that all litters were represented by at least 1 pup, with as few litters as possible represented by 2 pups (maintaining the litter as the experimental unit). Of these, 10 animals/sex/group were selected by the pathologist for neuropathological and brain morphometric evaluations on PND 72 such that measurements were recorded on homologous sections, the dimensions of the regions were comparable, as many litters as possible were represented, and same-sex littermates were not evaluated. All animals not selected for brain weight/neuropathology/morphometry were euthanized by carbon dioxide inhalation and necropsied on PND 72.

Subset B of 20 pups/sex/group was selected for evaluation of learning and memory (PND 22). Indicators of physical development (balanopreputial separation and vaginal patency) were also evaluated for all animals in Subset B. Following attainment of sexual developmental landmarks all animals in this Subset were euthanized by carbon dioxide inhalation and necropsied.

Subset C of 15 pups/sex/group was selected for brain weight and measurement evaluations on PND 21; of these, 10 pups/sex/group were selected by the pathologist for neuropathological and brain morphometric evaluations on PND 21 such that measurements were recorded on homologous sections, the dimensions of the regions were comparable, as many litters as possible were represented, and same-sex littermates were not evaluated. For brain morphometric evaluations, in some instances as few as 8 pups/sex/group were evaluated (as approved by the study pathologist) in a particular measurement, due to artifact changes in a given structure.

TABLE 1. Study Design

Experimental parameter	Demiditraz (PF-03814927) Dose (mg/kg/day)			
	0 (Vehicle)	5	15	100
Maternal animals				
No. of maternal animals assigned	25	25	25	25
FOB (GD 10, 15; LD 10, 20)	25	25	25	25
Offspring				
Detailed clinical/FOB (PND 4, 11, 21, 35, 45, and 60) A*	20/sex	20/sex	20/sex	20/sex
Motor activity (PND 13, 17, 21, and 61) A	20/sex	20/sex	20/sex	20/sex
Auditory startle habituation (PND 20 and 60) A	20/sex	20/sex	20/sex	20/sex
Learning and memory				
PND 22 B	20/sex	20/sex	20/sex	20/sex
PND 62 A	20/sex	20/sex	20/sex	20/sex
Brain weight and Brain measurements (length/width)				
PND 21 C	15/sex	15/sex	15/sex	15/sex
PND 72 A**	15/sex	15/sex	15/sex	15/sex
Neuropathology and Morphometrics				
PND 21 C	10/sex	10/sex	10/sex	10/sex
PND 72 A	10/sex	10/sex	10/sex	10/sex
Sexual Development**				
Balanopreputial separation (A, B)	20/sex	20/sex	20 /sex	20 /sex
Vaginal patency (A, B)	20/sex	20/sex	20/sex	20/sex

*The letters A, B, C designate the Subset group of offspring used for that experimental parameter.
** All F_1 animals in Subsets A and B, a total of 40/sex/group, were assessed for sexual development but these animals from both Subsets represented a total of 20 litters/group. Since litter is considered the statistical unit, each test group for these parameters of sexual development was considered as n = 20/sex.

All F_1 pups not selected for neuropathological or behavioral evaluations were euthanized by carbon dioxide inhalation and necropsied on PND 21. All surviving F_0 females with viable pups on lactation day 21 (PND 21) and those that did not deliver were also euthanized by carbon dioxide inhalation and necropsied.

5. **Dose selection rationale:** Dose levels were chosen based primarily on the results of a previous preliminary developmental neurotoxicity study (Beck, 2012, WIL-344067) which is summarized in Appendix A. Briefly, in this preliminary study, F_0 females were administered the test substance at dosage levels of 5, 15, 30, and 100 mg/kg/day once daily from gestation day 6 to lactation day 12. Clinical findings of hypoactivity, a flattened body, sitting with the head held low, hunched posture, rocking/lurching/swaying while ambulating, shallow

respiration, slightly drooping eyelids, and clear/yellow material on various body surfaces were noted at 15, 30, and/or 100 mg/kg/day. Lower mean body weights and/or body weight gains were noted at 30 and 100 mg/kg/day, with corresponding effects on mean food consumption at 100 mg/kg/day. In addition, increased pup mortality, decreased pup postnatal survival, and decreased pup body weights were noted for F_1 pups at 100 mg/kg/day. Additional information was obtained from a previous 2-generation study (Stump, 2009, WIL-344040), in which F_0 male and female rats were administered the test substance at dosage levels of 7.5, 50, and 150 mg/kg/day daily by oral gavage for at least 70 consecutive days prior to mating and continuing through 1 day prior to euthanasia, while F_1 offspring were administered the test substance beginning on PND 22 through 1 day prior to euthanasia. Increased gestation and estrous cycle lengths and pre-coital intervals, higher mean unaccounted-for sites and lower mean numbers of pups born were observed at 150 mg/kg/day. In addition, mortality and lower mean body weight gains and food consumption were observed at 150 mg/kg/day, and clinical findings (primarily CNS-related) were observed at >50 mg/kg/day. Furthermore, low mean birth weights, lower mean pup body weight gain, and decreased postnatal survival were observed at >50 mg/kg/day. Based on the aforementioned findings, dosage levels of 5, 15, and 100 mg/kg/day were selected for the current study. The selected route of administration for this study was oral (gavage) because this is a potential route of accidental human exposure.

6. **Dosage administration:** All doses were administered once daily to F_0 maternal animals orally by gavage, from gestation day 6 through lactation day 20, in a volume of 10 mL/kg of body weight/day. Dosing was based on the most recently recorded body weights. All animals were dosed at approximately the same time each day between approximately 1300 to 1500 hours daily.

7. **Dosage preparation and analysis:**

Dosing formulations were prepared at the test substance concentrations indicated as follows:

Group Number	*Treatment*	*Dosage Level (mg/kg/day)*	*Test Substance Concentration (mg/mL)*	*pH**
1	Vehicle	0	0	6.35
2	Demiditraz	5	0.5	8.28
3	Demiditraz	15	1.5	8.30
4	Demiditraz	100	10	8.29

* pH measurement of the first dosing formulations.

Formulations were prepared approximately weekly by mixing appropriate amounts of test substance with the vehicle suspension as single formulations for each dosage level, divided into aliquots for daily dispensation, and stored refrigerated. Prior to the start of the study, stability of the test substance in vehicle at concentrations of 0.1 and 100 mg/ml following up to 10 days of refrigerated and room temperature storage was established in a previous study (Bowman, 2007, WIL-344039).

Prior to the initiation of dose administration, samples for homogeneity and/or concentration determination were collected from the top, middle, and bottom strata of the first formulations prepared for dose administration (0, 0.5, 1.5, and 10 mg/mL). An aliquot of sufficient size for dosing a group of animals for 1 day was removed from the 0.5 and 10 mg/mL formulations and stored refrigerated for 10 days; following remixing for at least 30 minutes, samples were collected from the top and bottom strata of each aliquot for determination of resuspension

homogeneity. Samples for concentration analysis were collected weekly from the middle stratum of each dosing formulation (including the control group). One set of samples from the first and fourth weeks of dose administration was analyzed. The remaining samples were stored frozen (approximately -20°C) as back-up.

Results of Homogeneity analysis: Duplicate samples from the top, middle, and bottom strata of the formulations prepared on 4/21/11 at target test substance concentrations of 0.5 and 10 mg/ml (and middle strata only at the target concentration of 1.5 gm/mL) were analyzed to assess test substance dosing formulation homogeneity.

Dose Group/Strata	*Dose Conc.*	*Mean Analyzed Conc.*			
		mg/mL	*SD*	*Relative SD(%)*	*% of Target*
2/Top-Mid-Btm	0.5 mg/mL	0.508	0.012	2.5%	102
3/Mid	1.5 mg/mL	1.49	0.0016	0.11%	99.2
4/Top-Mid-Btm	10 mg/mL	10.1	0.17	1.7%	101

The homogeneity assessment met the WIL Research requirement, i.e., the RSD (relative standard deviation) for the mean concentration was <10% at a concentration within the acceptable limits of 85% to 115% of target concentration.

Results of Stability analysis: Representative aliquots of the 0.5 and 10 mg/ml formulations used for the homogeneity analysis above were stored refrigerated for 10 days, at which time the test substance was resuspended by stirring. Duplicate samples were collected from the the top and bottom strata of the aliquots and analyzed to assess 10-day resuspension homogeneity as an index of stability.

Nominal Concentration	*Actual Mean Concentration*			
of Formulation	*mg/mL*	*SD*	*Relative SD(%)*	*% Target*
0.5 mg/mL	0.441	0.011	2.4%	88.2
10 mg/mL	8.76	0.29	3.3%	87.6

The resuspension homogeneity assessment (index of stability) after 10 days refrigerator storage met the WIL Research requirement, i.e., the RSD for the mean concentration was ≤10%.

Results of Concentration analysis*:* Formulations used for dose administration were analyzed to assess test substance concentration acceptability.

Date of Preparation	*Dose Group*	*Mean Analyzed Concentration*			
	Concentration	*mg/mL*	*SD*	*Relative SD(%)*	*% of Target*
21 April 2011	**#1 - 0 mg/mL**	**----------Not Quantifiable----------**			
	#2 - 0.5 mg/mL	0.508	0.012	2.5%	102
	#3 - 1.5 mg/mL	1.49	0.0016	0.11%	99.2
	#4 - 10 mg/mL	10.1	0.17	1.7%	101
21 April 2011	**#1 - 0 mg/mL**	**----------Not Quantifiable----------**			
	#2 - 0.5 mg/mL	0.500	0.0082	1.6%	100
	#3 - 1.5 mg/mL	1.49	0.0067	0.45%	99.2
	#4 - 10 mg/mL	10.2	0.097	1.0%	102

The analyzed formulations used for dose administration met the WIL Research SOP requirement for concentration acceptability for suspension formulations, i.e., the analyzed concentration was 85% to 115% of the target concentration.

The analytical data indicated that the mixing procedure was adequate and that the difference between nominal and actual dosage to the study animals was acceptable.

C. OBSERVATIONS:

1. In-life observations:

a. Maternal animals: All rats were observed twice daily for moribundity and mortality. Individual clinical observations were recorded daily (at a time prior to test substance administration during the treatment period) for each Fo female from gestation day 0 until necropsy. Animals were also observed daily for signs of toxicity at the time of dosing and approximately 15-30 minutes and 2 hours following dose administration throughout the treatment period, except on the days of detailed clinical observations, i.e. FOB (GD 10 and 15; LD 10 and 20). The absence or presence of findings was recorded for individual animals. Females expected to deliver litters were also observed twice daily during the period of expected parturition and at parturition for dystocia (prolonged labor, delayed labor or other difficulties). The post-dosing observations were conducted by the same 9 biologists who were trained to recognize the effects of neurotoxicants.

To evaluate potential treatment-related effects on maternal care, the daily and post-dosing observations during lactation days 1-7 included a comment regarding the location of the dam with respect to her litter and amount of litter scattering using the following predefined descriptive criteria:
• Dam on pups
• Dam away from nest but actively tending to litter (e.g., retrieving pups or building the nest)
• Dam away from nest but actively eating, drinking, or grooming
• Dam away from the nest but not actively eating, drinking, or grooming and not tending to the litter (i.e., sleeping, resting on food jar)
• Litter not scattered (all pups in nest)
• Litter scattered with 1-3 pups outside nest
• Litter scattered with more than 3 pups outside nest
These observations were conducted by the same 5 biologists.

Detailed clinical observations, including parameters conducted in the home cage and outside of the home cage during handling and during a 2-minute open field observation period (as based on and containing components of the "Functional Observational Battery", FOB), were recorded for all F_0 females in each treatment group approximately 20 minutes following dose administration on gestation days 10 and 15 and on lactation days 10 and 20. Procedurally, the specific parameters observed in the home cage included: general body posture, tremors, convulsions, and arousal. Observation made during handling included: ease of removal from the cage, ease of handling animal in hand, salivation, fur appearance and coloration, lacrimation/chromodacryorrhea, respiratory rate/character, palpebral closure, mucous membranes/eye/skin color, piloerection, muscle tone, red/crusty deposits (eyes, nose, mouth), eye prominence, and

pupil response. Observations made during the 2-minute open field period included: arousal (beginning on gestation day 15), bizarre/stereotypic behavior, mobility, gait, urination/defecation, backing, convulsions/tremors, and grooming. It should be noted that forelimb and hindlimb grip strength testing was not included in the observational battery used for the F_0 females. Dosing on these testing days of the F_0 females was staggered to allow for the reasonable conduct of the detailed clinical observation assessments and to ensure that observations could be performed at the appropriate post-dosing time. The order of testing was counterbalanced across dose group, to the extent possible, using a computer-randomized procedure. In addition, testing was performed by the same 5 biologists, who were trained to recognize the effects of neurotoxicants and did not have knowledge of the animal's group assignment. The detailed clinical observations for the F_0 females were generally recorded as noted in the table below:

	DETAILED CLINICAL OBSERVATIONS
X	Signs of autonomic function, including: 1) Ranking of degree of lacrimation/chromodacryorrhea and salivation, with range of severity scores from none to severe 2) Presence or absence of piloerection and exophthalamus 3) Ranking or count of urination and defecation, including polyuria and diarrhea 4) Pupillary function scored as presence or absence of constriction of the pupil in response to light 5) Ranking of degree of palpebral closure with range from wide open to completely shut
X	Description, incidence, and ranked severity of any convulsions, tremors, abnormal mobility, or abnormal movements
X	Description and incidence of posture and gait abnormalities
X	Description and incidence of any unusual or abnormal behaviors, and ranked changes in level of arousal (beginning on gestation day 15), ease of handling, ease of removal from cage, excessive or repetitive actions (stereotypies), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance and color, grooming counts, coloration of mucous membrane, red or crusty deposits around the eyes, nose, or mouth, respiratory rate/character, and any other observations that may facilitate interpretation of the data.

More detailed descriptions of the scoring criteria used for each observation was included in an appendix E to the final report.

Individual maternal body weight data were recorded on gestation days 0, 6, 9, 12, 15, 18, and 20 and on lactation days 0 (when possible), 1, 4, 7, 11, 14, 17, and 21. Group mean body weight changes were calculated for each corresponding interval of gestation and lactation, and also for gestation days 6-20 and for lactation days 1-21. Individual maternal food consumption was recorded on gestation days 0, 6, 9, 12, 15, 18, and 20 and on lactation days 1, 4, 7, 11, 14, 17, and 21. Food intake was reported as g/animal/day and g/kg/day for the corresponding body weight change intervals.

All females were allowed to deliver naturally and rear their young to weaning (PND 21). During the period of expected parturition, the females were observed twice daily for initiation and completion of parturition and for signs of dystocia. Individual gestation length was calculated using the date delivery was first observed.

b. Offspring:

1) **Litter observations:** Beginning on the day parturition was initiated (PND 0), pups were sexed and examined for gross malformations, and the numbers of stillbirths and live pups were recorded. All pups were individually identified by tattoo markings on

the digits following completion of parturition. Pups were individually weighed on PND 1, 4, 7, 11, 14, 17, and 21 and at weekly intervals thereafter (Subsets A and B only) until necropsy, and whenever they were removed from their cages for behavioral testing (the latter offspring weights are maintained in the study records but not presented in the final report). Pups were individually sexed on PND 0 (when possible), 4, 11 and 21 and. Each litter was examined daily for survival and any adverse changes in appearance or behavior. Clinical observations for appearance, behavior including any abnormalities in nursing behavior, and overt signs of toxicity were performed on each pup on PND 1, 2, 3, and 4, twice weekly from PND 5 through 21 (on days of body weight collection), and at weekly intervals thereafter until euthanasia; observations were recorded prior to maternal dose administration, when appropriate. In addition, all pups were observed for the presence or absence of milk in the stomach on PND 1, 2, 3, and 4. The biologists performing the clinical observations were trained to recognize the effects of neurotoxicants.

Each dam and litter remained together until weaning on lactation day 21. On day 4 postpartum, all litters were standardized *by randomly selecting* within each litter a maximum of 8 pups/litter (4/sex/litter, when possible) to continue on study; culled pups were weighed, euthanized (i.p. sodium pentobarbital), and discarded. Selected pups retained the dam number and its original tattoo mark (e.g., 99999-01). Following weaning, each selected pup was uniquely identified by a metal ear tag displaying the identification number.

2) **Developmental landmarks:** Indicators of physical development (balanopreputial separation and vaginal patency) were evaluated for all F_1 selected animals in Subsets A and B beginning on postnatal day 35, male offspring were examined daily for balanopreputial separation. Beginning on postnatal day 25, female offspring were examined daily for vaginal lumen opening. The age of attainment of these landmarks, along with individual body weights on the day of attainment, were recorded.

3) **Postweaning observations:** After weaning on postnatal day 21, clinical observations for appearance, behavior, and overt signs of toxicity were performed on each surviving offspring (Subsets A and B) at weekly intervals until euthanasia. Individual offspring body weight data were also recorded weekly, on the day of attainment of sexual landmarks, and whenever they were removed from their cages for behavioral testing (the latter offspring weights are maintained in the study records but not presented in the final report).

4) **Neurobehavioral evaluations:** Observations and the schedule for those observations are summarized as follows from the report.

   a) **Detailed Clinical Observations (Functional Observational Battery; FOB):** Detailed clinical observations, including parameters conducted inside the home cage and outside of the home cage during handling and during a 2-minute open field observation period were recorded for 20 pups/sex/group in Subset A on PND 4, 11, 21, 35, 45, and 60, at a time prior to that of dosing of the dams. All assessments were conducted without knowledge of the animal's group assignment. The same 20 animals/sex/group were examined at each interval. Testing was counterbalanced across dose group and sex, to the extent possible,

using a computer-randomized procedure. Parameters evaluated were identical to those evaluated for the F_0 dams (see Section C.1.a above), with the following exceptions: (1) Post-weaning assessments (PND 21, 35, 45, and 60) included forelimb and hindlimb grip strength measurement. It should be noted that the final report provided no description of either the specific procedure or the equipment used for grip strength assessments. Also, the only 'control' data provided for grip strength consisted of historical control mean values for forelimb and hindlimb grip strength in gram units with ranges and standard deviation values for male and female rats at ages of PND 20, 21, 22, 35, 45 and 60; no positive control data for grip strength were provided. (2) The detailed clinical assessments of offspring in the present study did not include the recording of piloerection, fur appearance, palpebral closure, eye color, eye prominence, pupil response, mobility, gait, backing, and grooming on PND 4 and/or 11 since the pups' stage of development precluded assessment of those parameters on those days.

b) **Locomotor activity testing:** Locomotor activity was assessed for 20 rats/sex/group in Subset A on PND 13, 17, 21, and 61 between approximately 0830 to 1230 hours, such that testing was conducted prior to dosing of the dams. It was not specifically stated whether activity testing on PND 21 was conducted after the detailed clinical observations on that same day. Pups were selected for testing using a computer randomization procedure that selected an equal number of males and females across dose groups, to the extent possible, by age of the animal (up to 24 pups per 60-minute testing session). Locomotor activity testing was performed in a room equipped with a white-noise generation system set to operate at 70 ± 10 decibels (dB). Testing was conducted by biologists using an automated personal computer-controlled system that utilized a series of infrared photobeams surrounding an amber, plastic rectangular cage to quantify an animal's locomotor activity. Four-sided black plastic enclosures were used to surround the transparent plastic boxes and decrease the potential for distraction from extraneous environmental stimuli. The black enclosures rested on top of the photobeam frame and did not interfere with the path of the beams. Each animal was tested separately. Data were collected in 5-minute epochs (print intervals) and the test session duration was 60 minutes. These data were compiled as six 10-minute subintervals for tabulation. Data for ambulatory and total locomotor activity were tabulated. Total locomotor activity was defined as a combination of fine locomotor skills (i.e., grooming; interruption of a single photobeam) and ambulatory locomotor activity (e.g., interruption of 2 or more consecutive photobeams).

c) **Auditory startle reflex habituation:** Auditory startle response reflex habituation testing was performed on 20 rats/sex/group in Subset A (1 pup/sex/litter) on PND 20 and 60 using an automated system. Testing was conducted between approximately 0930 to 1230 hours (prior to dosing of the dams) and was performed following the detailed clinical observations on PND 60. Testing was counterbalanced across dose group, to the extent possible, using a computer-randomized procedure. The same animals were tested at each interval. Each compact cabinet was composed of high-quality laminate and medium-density fiberboard, and measured 10.9 x 14.0 x 19.5 inches. Each cabinet was equipped with an internal light, viewing lens, and a white-noise generation system. The

animal was placed in a rectangular enclosure of appropriate size, which was then placed into the isolation cabinet. Each enclosure was equipped with a motion sensor. Auditory startle response testing was performed in a room equipped with a white-noise generation system set to operate at 70 ± 10 dB. Note that the environmental conditions in the auditory startle response test room, including lighting, temperature and humidity were not provided. Each test session consisted of a 5-minute acclimation period with a 65 ± 5 dB broadband background white noise. The startle stimulus for each trial was a 115 ± 5 dB mixed-frequency noise burst stimulus, approximately 20ms in duration. Responses were recorded during the first 100ms following the onset of the startle stimulus for each trial. Each automated test session consisted of 50 trials, with an 8-second inter-trial interval. Startle response data were analyzed in 5 blocks of 10 trials each. Startle response measurements obtained were maximum response amplitude (MAX) in Newtons (N) and latency to maximum response amplitude (TMAX) in milliseconds (ms). The MAX measurement was the primary dependent variable analyzed, while TMAX was tabulated to verify consistency in measuring the primary startle response but was not statistically analyzed.

d) Learning and memory testing (Biel maze swimming trials): Learning, memory and swimming abilities were assessed using a water-filled 8-unit complex T-maze (Biel, 1940; WIL SOP T1-301) at two age periods with separate sets of animals. All data were recorded manually. The test interval beginning on PND 22 was conducted with 20 rats/sex/group from Subset B (1 pup/sex/litter) and the second test interval beginning on PND 62 used 20 rats/sex/group from Subset A (1 pup/sex/litter). Animals tested at PND 22 were not used for the second testing interval. The order of testing was counterbalanced across dose group, to the extent possible, using a computer-randomized procedure. Animals were placed in the maze and were required to traverse the maze and escape by locating a submerged platform. The time required to traverse the maze and the numbers of errors for all trials were manually recorded. An error was defined as any instance when the animal deviated from the correct channel with all 4 feet.

Each testing interval consisted of 3 phases that were conducted over 7 consecutive days. Phase 1 was an evaluation of swimming ability and motivation to escape from the maze, and was performed on day 1 of the Biel maze procedure. For this evaluation, animals were placed in a straight channel opposite the escape platform, and the time required for each animal to escape was recorded. Each animal was allowed 4 trials to evaluate swimming ability and motivation. For each trial, animals were allowed 2 minutes to complete the trial. At the end of each trial, the animal was immediately placed at the starting position for another trial until all 4 trials were complete. Phase 2 of the Biel maze procedure evaluated sequential learning. This evaluation was conducted on days 2-6 of the Biel maze procedure. Animals were allowed 2 trials per day for 2 days to solve the maze in path A (total 4 trials). Animals were then allowed 2 trials per day for 3 consecutive days (total 6 trials) to solve the maze in path B (reverse of path A). Phase 3 of the Biel maze procedure probed the animal for its memory to solve the maze when challenged in path A. This evaluation was conducted on day 7 of the Biel maze procedure. Each animal was allowed 2 trials to solve the maze in path A. For each trial in Phases 2 and 3, animals were allowed 3 minutes to solve the

maze. If an animal did not escape the maze within the allotted time, it was placed on the escape platform for up to 20 seconds, then removed from the maze. The minimum intertrial interval for Phases 2 & 3 was 1 hour.

Biel maze data were evaluated as the mean time to escape over trials for each of the 3 phases (i.e., swimming ability and motivation, sequential learning, and memory) of the Biel maze procedure. Also, the numbers of errors committed during Phases 2 and 3 were evaluated.

It should be noted that details of the learning/memory test equipment, including dimensions of the maze and escape platform, water depth, water temperature, and the environmental conditions in the testing room (including type of lighting, temperature, humidity) were not provided in the final report.

5) Cholinesterase determination: N/A

6) Pharmacokinetic data: N/A

2. Postmortem observations:

a. Maternal animals: All surviving females with viable pups on lactation day 21 and any that did not deliver (post-mating day 25) were euthanized by carbon dioxide inhalation. A gross necropsy was performed for each of these females; the thoracic, abdominal, and pelvic cavities were opened and the contents were examined. For females that delivered, the numbers of former implantation sites (the attachment site of the placenta to the uterus) were recorded. The number of unaccounted-for implantation sites was calculated by subtracting the number of pups born from the number of implantation sites observed. For females that failed to deliver, a pregnancy status was determined, and specific emphasis was placed on anatomic or pathologic findings that may have interfered with pregnancy. Tissues were preserved in 10% neutral-buffered formalin for possible future histopathologic examination only as deemed necessary by the gross findings. The carcass of each female was then discarded. Uteri with no macroscopic evidence of implantation were opened and subsequently placed in a 10% ammonium sulfide solution for detection of early implantation loss.

b. Offspring:

1) Gross Pathology Assessment:

Intact offspring found dead or euthanized in extremis (by intraperitoneal injection of sodium pentobarbital) from PND 0 to 4 were necropsied using a fresh dissection technique, which included examination of the heart and major vessels. The stomachs were examined for the presence of milk on PND 0 and 1. A detailed gross necropsy was performed on any pup that was found dead after PND 4. Tissues were saved in 10% neutral-buffered formalin for possible histopathological examination only as deemed necessary by the gross findings. In addition, 2 pups (1 each in control and 5 mg/kg/day group) were noted with bone fractures and retained in 100% ethyl alcohol for possible future examination. The carcasses of all other pups were discarded.

On PND 21, all F_1 animals not selected for postweaning evaluations were euthanized by carbon dioxide inhalation and subjected to a gross examination. The necropsy included examination of the external surface, all orifices and the cranial, thoracic, abdominal, and pelvic cavities, including viscera. Tissues were retained only as deemed necessary by the gross findings.

All Subset B offspring scheduled for euthanasia after completion of the learning and memory evaluations (PND 22) and subsequent acquisition of sexual developmental landmarks (20 rats/sex/group) and those offspring in Subset A that were scheduled for euthanasia on PND 72 but not allocated for neuropathology/morphometry/brain weight measurements (5 rats/sex/group) were euthanized by carbon dioxide inhalation and subjected to a gross pathology examination. The necropsy included examination of the external surface, all orifices and the cranial, thoracic, abdominal, and pelvic cavities, including viscera. Organ and tissue samples were preserved in 10% neutral-buffered formalin as deemed necessary by the gross findings. The carcass of each animal was then discarded.

2) Macroscopic Neuropathology Examination (Subset C) and Brain Measurements (Subsets A and C):

On PND 21, the 15 rats/sex/group in Subset C (1 pup/sex/litter), when possible, were macroscopically examined for neuropathology. On PND 72 (termination of the study), a total of 15 rats/sex/group (1 male and/or 1 female from each litter) was randomly selected from those pups in Subset A that were dedicated to behavioral evaluation (locomotor activity, auditory startle response, and learning/memory tests) and physical development (body weights and sexual landmarks). All animals were anesthetized by an intraperitoneal injection of sodium pentobarbital and perfused in situ with fixative (4% paraformaldehyde in 0.1 M phosphate buffer) as described in WIL Research's SOPs. The head was placed in a container of 10% neutral-buffered formalin. Following a minimum of 36 hours, the whole brains (including olfactory bulbs) were removed from the skull, weighed, and the dimensions (length [excluding olfactory bulbs] and width) were recorded for all Subset A (PND 72) and C (PND 21) animals. The timing of all brain dissections was kept as constant as possible (approximately 48 hours following perfusion). Any abnormal coloration or lesions of the external brain and spinal cord were recorded. On PND 21, brains from all animals were retained for microscopic examination; at study termination (PND 72), the brains, as well as peripheral nervous system tissues, were dissected and preserved for microscopic examination.

3) Microscopic Neuropathology Evaluation:

Brains from 10 pups/sex/group selected for microscopic evaluation on PND 21 and 72, each, were prepared for a qualitative histopathological examination. At each age period, neuropathological examination was performed initially on the brains of 10 pups/sex/group from the control and high-dose groups. Upon review and recommendation by the study pathologist, sections from the brains of animals in all treatment groups were examined to further evaluate findings that were limited to high-dose group animals. Approximately 24 sections (5 microns thick) at 8 levels from all major brain regions (including olfactory bulbs, cerebral cortex, hippocampus,

basal ganglia, thalamus, hypothalamus, midbrain, cerebellum, pons, and medulla oblongata) were examined from the selected animals. In addition to the qualitative histopathological evaluation, a simple morphometric analysis, as described below, was performed on 10 rats/sex in all groups (no more than 1 rat/sex/litter in each group) on PND 21 and 72, each. Specific measurements taken were determined by the pathologist, but included at least 2 measurements from the neocortical and hippocampal areas, and 1 measurement from the cerebellar area. For brain morphometric evaluations, in some instances as few as 8 pups/sex/group were evaluated (as approved by the study pathologist) in a particular measurement, due to artifact changes in a given structure or to avoid evaluation of non-homologous sections.

Peripheral nervous system tissues from the offspring perfused in situ at study termination (PND 72, Subset A) were processed for neuropathological evaluation and microscopically examined for 10 rats/sex in the control and high-dose groups. These tissues included spinal cord at cervical and lumbar levels, lumbar and cervical dorsal root ganglia and dorsal/ventral root fibers, sciatic nerves, sural nerves, tibial nerves, optic nerves, and eyes with retina.

All tissues for a single sex were trimmed by 1 technician, alternating between groups such that all dose groups were evenly distributed across the days of trimming. The central and peripheral nervous system tissues were placed into properly labeled cassettes for processing. The cassettes were loaded onto the tissue processor such that each dose group and sex was equally represented in each run, to the extent possible. The central nervous system tissues were embedded in paraffin, and the peripheral nervous system tissues were embedded in glycol methacrylate. Tissues were prepared for histopathological evaluation by sectioning and staining with hematoxylin and eosin. Sectioning was conducted by 2 technicians (1 technician for tissues from males and 1 technician for tissues from females; the same technicians that trimmed tissues, as described above) using the same microtome and water bath. [A slight procedural discrepancy was noted regarding the sectioning of brain tissues. The Pathology Report (Appendix F, page 559 of the final report) stated that “To avoid artifacts in morphometric measurements, a single histotechnologist was responsible for sectioning all brains from a particular age ..”. In the description of procedures in the final report itself (page 61) it was stated, as noted above, that “Sectioning was conducted by 2 technicians (1 technician for tissues from males and 1 technician for tissues for females…)”]. The tissue blocks were arbitrarily selected, alternating between groups using a round robin approach (i.e., Group 1, Group 2, Group 3, Group 4, etc.), such that all groups were evenly distributed across the days of sectioning. Missing tissues were identified as not found at necropsy, lost at necropsy, lost during the processing, or other designations as appropriate.

Following completion of the tissue evaluation by the study pathologist, a peer review evaluation was performed by an independent pathologist and the results presented in the final report provided a consensus of the study pathologist and peer review pathologist.

4) Morphometric Analysis:

Quantitative examinations of the brains from the selected PND 21 and PND 72 offspring (10 pups/sex/group each) were conducted using the Pax-It™ (MIS, Inc., Franklin Park, IL) image-capturing computer system and software (version 4.0) and included the following. Morphometric analysis was performed on the brains of the same animals selected for microscopic examination. Slides from all animals that were perfused for brain weights/measurements were provided to the pathologist. The pathologist selected sections from 10 pups/sex/group for morphometric analysis; the same pups were used for collection of all measurements, although in some instances as few as 8 pups/sex/group were included (as approved by the study pathologist) in a particular measurement, due to artifactual changes in a given structure. Simple linear morphometric measurements were obtained from homologous sections of the brain at 3 levels. Level 1, analyzed for PND 22 animals, was a coronal section obtained just rostral to the genu of the corpus callosum and contained the following major structures: cerebral cortex, corpus callosum, basal ganglia (caudate putamen, nucleus accumbens), and lateral ventricles. Two bilateral measurements were obtained at this level: the vertical height of the hemisphere just medial to the lateral ventricle (SI), and the vertical height of the cerebral cortex from the apex of the corpus callosum/cingulum to the dorsal surface of the hemisphere (S2). Level 2 was used for the PND 72 rat brains because of difficulties with homology at Level 1 for some of these animals. Level 2, analyzed for PND 72 animals, was at the level of the optic chiasm near the widest part of the anterior commissure. Structures included at this level include the cerebral cortex (cingulate, motor, piriform), the basal ganglia (caudoputamen), the preoptic nuclei, and the lateral and third ventricles. Measurements taken at Level 2 were similar to those at Level 1, namely, 2 bilateral measurements: the greatest perpendicular height of a hemisphere, and the vertical height from the apex of the callosum/cingulum to the dorsal surface of the hemisphere. Level 3 was a coronal section obtained just rostral to the infundibular recess and contained the following major structures: cerebral cortex, corpus callosum, basal ganglia, hippocampus/dentate hilus, thalamus, hypothalamus, and lateral and third ventricles. Three bilateral measurements were taken at this level: the radial thickness of the cortex, the vertical height between the layers of the hippocampal pyramidal neurons along a line that passed through the termination of the dorsal limb of the dentate hilus, and the vertical height of the dentate hilus measured at the termination of the ventral limb. Level 5 was a sagittal section of the cerebellum and medulla oblongata, taken slightly lateral to the midline, and contained the following major structures: cerebellum, medulla oblongata (pyramidal tract, trapezoid body, medial longitudinal fasciculus), and fourth ventricle. Single measurements of the height of the cerebellum and of the thickness of the base of cerebellar lobule no. 9 were obtained at this level. For purposes of reporting, the measurements from the right and left hemispheres, where appropriate, were combined to obtain a mean overall measurement for that structure. Levels 1, 3, and 5 were measured for PND 21 rat pups. Levels 2, 3, and 5 were measured for PND 72 rats. Sections for morphometry were homologous (within at least approximately 250 microns of each other). Measurements were not taken on sections that were not homologous or on sections that had artifacts (e.g., tissue separations) that would compromise the measurement lengths.

D. DATA ANALYSIS:

1. Statistical analyses:

Analyses Conducted by WIL Research (Performing Laboratory)
All statistical tests were performed using the WIL Toxicology Data Management System (WTDMS) unless otherwise noted. Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each test substance-treated group to the control group by sex. The litter was used as the statistical unit for these analyses.

Mean maternal and offspring body weights and body weight changes, maternal food consumption, gestation lengths, former implantation sites, unaccounted-for sites, numbers of pups born, live litter sizes, day of attainment of balanopreputial separation or vaginal patency, body weight on the day of attainment, brain weights relative to final body weight of F_1 pups, and continuous detailed clinical observation data were subjected to a parametric one-way ANOVA to determine intergroup differences[1]. If the ANOVA revealed significant ($p<0.05$) intergroup variance, Dunnett's test was used to compare the test substance-treated groups to the control group. Mean litter proportions (percent per litter) of pup viability and males per litter were subjected to the Kruskal-Wallis nonparametric ANOVA test to determine intergroup differences. If the ANOVA revealed significant ($p<0.05$) intergroup variance, Dunn's test was used to compare the test substance-treated groups to the control group. Detailed clinical observation data which yielded scalar and descriptive data and qualitative histopathological findings were analyzed using Fisher's Exact Test.

Analyses Conducted by BioSTAT Consultants, Inc.
All analyses were conducted using SAS version 9.2 (SAS Institute, Inc., 2002-2008), or higher, software. Litter was used as the statistical unit for all F_1 pre-weaning and immediate postweaning behavioral testing but was not used as the statistical unit for adult behavioral testing.

Motor Activity
Motor Activity: F_1 Pup Motor Activity (Within Session) – PND 13, 17, and 21
The analysis in this section evaluated the effect of treatment compared to control for cumulative activity for a session and also evaluated if there was a treatment effect on the pattern of habituation within a session. Total and ambulatory counts from six 10-minute time intervals were analyzed, by session, with a repeated measures analysis of variance (RANOVA). Factors in the model included: random effects LITTER; fixed effects treatment (TRT), SEX, and TIME interval; interaction terms TRT*SEX, TRT*TIME, and TRT*SEX*TIME. The first examination was whether the TRT*SEX and/or TRT*SEX*TIME interaction was significant. If either interaction was significant, the analysis was conducted, by sex, with a RANOVA. Factors in the model included: fixed effects TRT and TIME and the interaction term TRT*TIME. The covariance structure for the RANOVA model was selected by comparing the corrected Akaike's Information Criteria (AICC). The Kenward-Rogers adjustment for denominator degrees of freedom

1 Fo female data obtained from nongravid animals were excluded from statistical analyses following the mating period.

wa applied. In the final RANOVA model, evaluations were conducted as follows: to evaluate cumulative activity across the session as a whole, the TRT main effect was evaluated and, if significant, Dunnett's test was used to compare each treated group with the control. In addition, if the TRT*TIME interaction was significant, pairwise comparisons of the interaction term were made for each individual treated group with the control group through linear contrasts. To adjust for multiple comparisons, the pairwise comparisons were made at the 0.02 significance level.

Motor Activity: F_1 Pup Motor Activity (Across Sessions) – PND 13, 17, and 21
The focus of this analysis was to determine if there was an interaction between treatment and the ontogenic pattern of cumulative motor activity across PND 13, 17, and 21. Cumulative total and ambulatory counts from six 10-minute SESSION intervals were analyzed with a RANOVA. Factors in the model included: random effects LITTER; fixed effects TRT, SEX, and SESSION; interaction terms TRT*SEX, TRT*SESSION, and TRT* SEX*SESSION. The first examination was whether the TRT*SEX and/or TRT*SEX*SESSION interaction was significant. If either interaction was significant, the analysis was conducted, by sex, with a RANOVA. Factors in the model included: fixed effects TRT and SESSION and the interaction term TRT*SESSION. The covariance structure for the RANOVA model was selected by comparing the AICC. The Kenward-Rogers adjustment for denominator degrees of freedom was applied. In the final RANOVA model, evaluations were conducted as follows: if the TRT*SESSION interaction was significant, pairwise comparisons of the interaction term were made for each individual treated group with the control group through linear contrasts. To adjust for multiple comparisons, the pairwise interaction comparisons were made at the 0.02 significance level. If the TRT*SESSION interaction was not significant, no further analyses were conducted.

Motor Activity: Adult Motor Activity (Within Session) – PND 61
The analysis in this section evaluated the effect of treatment compared to control for total activity for this session and also evaluated if there was a treatment effect on the pattern of habituation within this session. Total and ambulatory counts from six 10-minute time intervals were analyzed with a RANOVA. Factors in the model included: fixed effects TRT, SEX, and TIME interval; interaction terms TRT*SEX, TRT*TIME, and TRT* SEX*TIME. The first examination was whether the TRT* SEX and/or TRT*SEX*TIME interaction was significant. If either interaction was significant, the analysis was conducted, by sex, with a RANOVA. Factors in the model included: fixed effects TRT and TIME and the interaction term TRT*TIME. The covariance structure for the RANOVA model was selected by comparing the AICC. The Kenward-Rogers adjustment for denominator degrees of freedom was applied. In the final RANOVA model, evaluations were conducted as follows: to evaluate cumulative activity across the session as a whole, the TRT main effect was evaluated and, if significant, Dunnett's test was used to compare each treated group with the control. In addition, if the TRT*TIME interaction was significant, pairwise comparisons of the interaction term were made for each individual treated group with the control group through linear contrasts. To adjust for multiple comparisons, the pairwise interaction comparisons were made at the 0.02 significance level.

Auditory Startle Response
Maximum response amplitude in Newtons (MaxN) was the primary dependent variable

statistically analyzed. Latency to maximum response amplitude in milliseconds (Tmax) was tabulated as a quality check for consistent selection of peak amplitude of the primary startle response but was not statistically analyzed.

Auditory Startle: F_1 Pup Auditory Startle Response (Within Session) – PND 20
MaxN was analyzed with a RANOVA. Factors in the model included: random effects LITTER; fixed effects TRT, SEX, and TRIAL block; interaction terms TRT*SEX, TRT*TRIAL, and TRT*SEX*TRIAL. The primary focus of this analysis was on the effect of treatment on the pattern of habituation within the session following repeated trials. The analysis in this section also evaluated the effect of treatment compared to control for MaxN (peak amplitude) across the session as a whole. The first examination was whether the TRT* SEX and/or TRT*SEX*TRIAL interaction was significant. If either interaction was significant, the analysis was conducted, by sex, with a RANOVA. Factors in the model included: fixed effects TRT and TRIAL and the interaction term TRT*TRIAL. The covariance structure for the RANOVA model was selected by comparing the AICC. The Kenward-Rogers adjustment for denominator degrees of freedom was applied. In the final RANOVA model, evaluations were conducted as follows: to evaluate MaxN across the session as a whole, the TRT main effect was evaluated and, if significant, Dunnett's test was used to compare each treated group with the control. In addition, if the TRT*TRIAL interaction was significant, pairwise comparisons of the interaction term were made for each individual treated group with the control group through linear contrasts. To adjust for multiple comparisons, the pairwise interaction comparisons were made at the 0.02 significance level.

Auditory Startle: Adult Auditory Startle Response (Within Session) – PND 60
MaxN was analyzed with a RANOVA. Factors in the model included: fixed effects TRT, SEX, and TRIAL block; interaction terms TRT* SEX, TRT*TRIAL, and TRT*SEX*TRIAL. The primary focus of this analysis was the effect of treatment on the pattern of habituation within the session following repeated trials. The analysis in this section also evaluated effect of treatment compared to control for MaxN (peak amplitude) across the session as a whole. The first examination was whether the TRT* SEX and/or TRT*SEX*TRIAL interaction was significant. If either interaction was significant, the analysis was conducted, by sex, with a RANOVA. Factors in the model included: fixed effects TRT and TRIAL and the interaction term TRT*TRIAL. The covariance structure for the RANOVA model was selected by comparing the AICC. The Kenward-Rogers adjustment for denominator degrees of freedom was applied. In the final RANOVA model, evaluations were conducted as follows: to evaluate MaxN across the session as a whole, the TRT main effect was evaluated and, if significant, Dunnett's test was used to compare each treated group with the control. In addition, if the TRT*TRIAL interaction was significant, pairwise comparisons of the interaction term were made for each individual treated group with the control group through linear contrasts. To adjust for multiple comparisons, the pairwise interaction comparisons were made at the 0.02 significance level.

Biel Water Maze
The analysis of time to escape and number of errors was conducted with a RANOVA for each phase separately (i.e., Learning Phase A, Learning Phase B, and Memory Phase A). The focus of the statistical analyses was the effect of treatment on learning (or memory) within each phase.

Water Maze: F_1 Pup Biel Water Maze – PND 22
The analysis of time to escape and number of errors was conducted with a RANOVA. Factors in the model included: random effects LITTER; fixed effects TRT, SEX, and TRIAL; interaction terms TRT*SEX, TRT*TRIAL, and TRT* SEX*TRIAL. The first examination was whether the TRT* SEX and/or TRT*SEX*TRIAL interaction was significant. If either interaction was significant, the analysis was conducted, by sex, with a RANOVA. Factors in the model included: fixed effects TRT and TRIAL and the interaction term TRT*TRIAL. The covariance structure for the RANOVA model was selected by comparing the AICC. The Kenward-Rogers adjustment for denominator degrees of freedom was applied. In the final RANOVA model, evaluations were conducted as follows: if the TRT*TRIAL interaction was significant, pairwise comparisons of the interaction term were made for each individual treated group with the control group through linear contrasts. To adjust for multiple comparisons, the pairwise interaction comparisons were made at the 0.02 significance level. If the TRT*TRIAL interaction was not significant, the TRT main effect was evaluated and, if significant, Dunnett's test was used to compare each treated group with the control.

Water Maze: Adult Biel Water Maze – PND 62
The analysis of time to escape and number of errors was conducted with a RANOVA. Factors in the model included: fixed effects TRT, SEX, and TRIAL; interaction terms TRT*SEX, TRT*TRIAL, and TRT* SEX*TRIAL. The first examination was whether the TRT*SEX and/or TRT*SEX*TRIAL interaction was significant. If either interaction was significant, the analysis were conducted, by sex, with a RANOVA. Factors in the model included: fixed effects TRT and TRIAL and the interaction term TRT*TRIAL. The covariance structure for the RANOVA model was selected by comparing the AICC. The Kenward-Rogers adjustment for denominator degrees of freedom was applied. In the final RANOVA model, evaluations were conducted as follows: if the TRT*TRIAL interaction was significant, pairwise comparisons of the interaction term were made for each individual treated group with the control group through linear contrasts. To adjust for multiple comparisons, the pairwise interaction comparisons were made at the 0.02 significance level. If the TRT*TRIAL interaction was not significant, the TRT main effect was evaluated and, if significant, Dunnett's test was used to compare each treated group with the control.

Water Maze: Straight Channel Swimming Ability – Both Test Intervals
Mean escape times for the last 3 of the 4 trials from the straight channel for each testing interval were subjected to a parametric one-way ANOVA to determine intergroup difference. If the results of the ANOVA were significant ($p<0.05$), Dunnett's test (1964) was applied to the data to compare the treated groups to the control group.

Morphometry
Males and females were analyzed separately. The endpoints were analyzed by sets of related brain measures as described below:
Set 1: SI, S2, S3 - measurements of the rostral cerebrum
Set 2: S4, S5 - measurements of the hippocampus
Set 3: S7, S8 - measurements of the cerebellum
Set 4: Brain weight, brain length, brain width
Each set of related brain measures was analyzed using a multivariate analysis of variance

(MANOVA) with treatment group (TRT) as a fixed factor. If TRT was statistically significant, linear contrasts, as part of the MANOVA across all dependent variables were used to test for pairwise differences between each treated group and control. The Type I error rate was corrected for multiple comparisons between the control group and each of the treated groups by taking the original Type I error rate of 0.05 and dividing that rate by the square root of the number of comparisons. If TRT was not significant, no additional statistical analysis was conducted.

2. Indices:

a. Reproductive indices: During the period of expected parturition, the females were observed twice daily for initiation and completion of parturition and for signs of dystocia. Individual gestation length was calculated using the the day on which evidence of mating was identified (gestations day 0) and the date delivery was first observed. Beginning on the day parturition was initiated (PND 0), pups were sexed and examined for gross malformations, and the numbers of stillbirths and live pups were recorded. At necropsy, for females that delivered, the numbers of former implantation sites (the attachment site of the placenta to the uterus) were recorded. The number of unaccounted-for implantation sites was calculated by subtracting the number of pups born from the number of implantation sites observed. For females that failed to deliver, a pregnancy status was determined, and specific emphasis was placed on anatomic or pathologic findings that may have interfered with pregnancy. Uteri with no macroscopic evidence of implantation were placed in a 10% solution of ammonium sulfide for detection of early implantation loss.

b. Offspring viability indices: The following viability (survival) indices were calculated as 'litter parameters' from lactation records of litters in the study:

- Mean Live Litter Size = Total no. of viable pups PND0 / No. of litters with viable pups PND0

- Postnatal Survival Between Birth and PND 0 or PND 4 [Pre-selection] (% per litter) = {Sum of (Viable pups per litter on PND0 or PND 4[Pre-selection] / No. of pups born per Litter)* / No. of litters per group} x 100

- Postnatal Survival for All Other Intervals (% per litter) = {Sum of (Viable pups per litter at end of interval N / Viable pups per litter at start of interval N)** / No. of litters per group} x 100

* A pup that was euthanized due to mechanical injury was excluded from pup viability calculations
** Where N – PND 0-1, 1-4 [Pre-selection], 4 [Post-selection]-7, 7-14, 14-21 or 4 [Post-selection]-21

3. Positive and Historical Control Data:

a. Positive Control Data:

Auditory Startle Positive Control Study # WIL-99418, conducted November 2006 – June 2008 by WIL Research Laboratories, LLC:

The objective of this study was to demonstrate the ability of the Kinder Scientific Startle Monitor System (used in MRID 48766703) (Kinder Scientific, LLC, Poway, CA, USA) to detect reductions in acoustic startle of weanling and adult rats in response to positive control treatment. Chlorpromazine, a low-potency typical antipsychotic drug known to produce decreases in startle response, was administered by subcutaneous injection at dosage levels of 2 or 10 mg/kg at postnatal day (PND) 20 or PND 60 to male and female Sprague-Dawley Crl:CD (SD) rats approximately 45 minutes prior to the neurobehavioral assessment. For PND 20 and 60 male and female rats, chlorpromazine treatment resulted in a dose dependent decrease in the maximum force (MAX) across all blocks of trials, with no effect on habituation. In general, the mean MAX values across all trial blocks for PND 20 and PND 60 rats was decreased compared to controls. There was also a trend of increased time until maximum force was achieved (TMAX) following chlorpromazine treatment in PND 20 and PND 60 rats. The acoustic startle response data (i.e., latency and startle force) generated in this study are consistent with previously reported effects of chlorpromazine on startle behavior. Therefore, the study report for this positive control study provided sufficient information to conclude that this study was appropriately designed and conducted and demonstrated that WIL Research Laboratories, LLC can detect treatment related reductions in the acoustic startle response in weanling and adult rats using the Kinder Scientific Startle Monitor System. However, this study did not demonstrate the ability to detect changes in acoustic startle response habituation using this monitoring system.

Auditory Startle Positive Control Study # WIL-99440, conducted October 2007 – September 2008 by WIL Research Laboratories, LLC:

The objective of this study was to demonstrate the ability of the Kinder Scientific Startle Monitor System (used in MRID 48766703) to detect increases in the acoustic startle response of weanling and adult rats in response to positive control treatment. Specifically, nicotine, S(-)8-Hydroxy-DPAT hydrobromide (8-OH-DPAT; a serotonin 1A receptor agonist able to modulate serotonergic transmission), and amphetamine were delivered to male and female Sprague-Dawley Crl:CD(SD) rats prior to the startle response assessment (N = 20 animals/sex for all treatment groups) . Nicotine was administered by intraperitoneal injection at dosage levels of 0, 0.06, 0.25 or 1 mg/kg on postnatal day (PND) 20 and 60; 8-OH-DPAT was administered by subcutaneous injection at dosage levels of 0, 0.2 and 2 mg/kg on PND 20 only; and amphetamine was administered by intraperitoneal injection at dosage levels of 0, 1 or 5 mg/kg on PND 60 only. For PND 20 nicotine-treated males and females, there was a trend of nicotine increasing the mean maximum response force, whereas in the PND 60 nicotine-treated males and females, the effects of nicotine on mean maximum force (MAX) were minimal. For both male and female PND 20 and 60 rats, habituation to the startle stimulus occurred over the five trial blocks. The 8-OH-DPAT treated male and female rats at the 0.2 and 2 mg/kg dose levels showed a significant increase in mean MAX generated as well as an increase in the startle response over the five trial blocks. Amphetamine-treated PND 60 male and female rats, specifically at 5 mg/kg, showed an increase in the mean MAX generated over all trial blocks, with a stronger startle habituation profile compared with controls being present over the individual trial blocks. The startle response data (i.e., latency and maximum force) generated using the Kinder Scientific Startle Monitor System are consistent with the previously reported effects of nicotine, 8-OH-DPAT and amphetamine on startle behavior. Therefore, the study report for this positive control study provided sufficient information to conclude that this study was appropriately designed and conducted and demonstrated that WIL Research Laboratories, LLC can detect treatment related increases in the acoustic startle response as well as variations in startle habituation in weanling and adult

rats using the Kinder Scientific Startle Monitor System.

Locomotor Activity Positive Control Study # WIL-99435, conducted October 2007-June 2008 by WIL Research Laboratories, LLC:
The objective of this study was to demonstrate the ability of the Kinder Scientific Motor Monitor System (used in MRID 48766703) (Kinder Scientific, LLC, Poway, CA, USA) to detect decreases in activity in rats during postnatal and adult periods of development in response to positive control treatment. Haloperidol was administered at dosage levels of 0.05, 0.1, and 0.5 mg/kg by a single intraperitoneal injection 30 minutes prior to behavioral testing on postnatal day (PND) 13, 17, 21 or 61 (N = 20 animals/sex for all treatment groups). Male and female Sprague-Dawley Crl:CD (SD) rats were used for assessment of locomotor behavior with the Kinder Scientific Motor Monitor System. Overall, haloperidol caused a dose-dependent decrease in locomotor activity (total activity counts and ambulatory counts) for both male and female rats at PND 13, 17, 21 and 61. Habituation was noted in PND 17, 21, and 61, but not in PND 13, males and females, was also disrupted by treatment. The data also detected the well-established developmental profile for locomotor activity over the PND 13 – 21 period. The motor activity data (i.e. total activity and ambulatory activity) generated in this study are consistent with the previously reported effects of haloperidol on motor behavior. Therefore, the study report for this positive control study provided sufficient information to conclude that this study was appropriately designed and conducted and demonstrated that WIL Research Laboratories, LLC can accurately profile the normal development of locomotor activity over the second and third weeks of postnatal development and and reliably detect treatment related reductions in the locomotor activity in postnatal, weanling and adult animals using the Kinder Scientific Motor Monitor System.

Locomotor Activity Positive Control Study # WIL-99441, conducted October 2007-May 2008 by WIL Research Laboratories, LLC:
The objective of this study was to demonstrate the ability of the Kinder Scientific Motor Monitor System (used in MRID 48766703) to detect increases in activity in rats during postnatal and adult periods of development in response to positive control treatment. Nicotine and amphetamine were administered to male and female Sprague-Dawley Crl:CD rats 3 and 15 minutes, respectively, prior to assessment of locomotor behavior with the Kinder Scientific Motor Monitor System (N = 20 animals/sex for all treatment groups except for the female-only PND 61 control and 0.2 mg/kg amphetamine groups). Nicotine was administered at a dosage level of 0.1 or 0.5 mg/kg by a single intraperitoneal injection to neonatal rats on postnatal days (PND) 13, 17 and 21. Amphetamine was administered at a dosage level of 1 or 3 mg/kg by a single intraperitoneal injection to young adult rats (male and female) on PND 61 and at a dosage level of 0.2 or 1 mg/kg to young adult PND 61 females. Nicotine caused an increase in locomotor activity (mean total and ambulatory activity) in male and female neonatal rats at PND 13, 17 and 21. The habituation profile was evident in the PND 17 and 21 males and females. In a similar manner, amphetamine stimulated locomotor behavior in male and female rats at PND 61, causing both total and ambulatory activity to increase. The subsequent female-only groups (0.2 and 1 mg/kg amphetamine) exhibited a dose response with increased total and ambulatory activity compared to controls. In the PND 61 males and females (given 1 or 3 mg/kg amphetamine), the habituation profile was only present in the control and 3 mg/kg amphetamine treatment groups, whereas the habituation profile was present in the female-only PND 61 control and 0.2 mg/kg amphetamine groups. The motor activity data (i.e. total activity and ambulatory activity) generated in this study are consistent with previously reported effects of nicotine

and amphetamine on motor behavior. Therefore, the study report for this positive control study provided sufficient information to conclude that this study was appropriately designed and conducted and demonstrated that WIL Research Laboratories, LLC can reliably detect treatment related reductions in locomotor activity in postnatal, weanling and adult animals using the Kinder Scientific Motor Monitor System.

Learning and Memory Testing (Biel Water Maze) Positive Control Study # WIL-99438, conducted October 2007-July 2008 by WIL Research Laboratories, LLC:
The objective of this study was to evaluate the ability of the Biel water maze learning and memory testing paradigm (used in MRID 48766703) to detect changes in cognitive function in Developmental Neurotoxicity studies. The Biel water maze 7-day testing paradigm was used to assess learning and memory in this positive control study: specifically, on day 1 four trials were given in a straight alley of the maze to assess swimming ability; on days 2 and 3 a total of 4 trials (2 trials per day) were given to assess learning Path A; on days 4 through 6 a total of 6 trials (2 trials per day) were given to assess learning Path B (the reverse of Path A); and on day 7 two trials were given to assess memory of Path A. Animals were allowed a maximum of 3 minutes to escape from the maze, after which they were removed; the minimum inter-trial interval for testing was 1 hour. This procedure was virtually identical to that used in the current study, (MRID 48766703) except that in the main study animals that did not complete the maze within 3 minutes were not immediately removed but were placed on the escape platform for a maximum of 20 seconds. Scopolamine, the positive control agent, was administered at dosage levels of 0, 0.5 and 1.5 mg/kg by intraperitoneal injection twice daily to male and female Sprague-Dawley Crl:CD(SD) rats from PND 22-28 (N = 20 animals/sex for all treatment groups). On the first day of testing (PND 22), doses were administered approximately 30 minutes prior to the first straight-channel swim trial and no sooner than 3 hours after the first daily treatment. On subsequent days (PND 23-28), doses were administered 30 minutes prior to each trial. Scopolamine administration, in general, resulted in impaired learning and memory (i.e., increased latency to escape and number of errors in the Biel water maze) in a dose dependent manner in both male and female treatment groups (0.5 and 1.5 mg/kg). Specifically, latency to escape the Biel maze was increased in both sexes in Path A and also in Path B following Scopolamine treatment (0.5 and 1.5 mg/kg). Likewise, error numbers were also increased in Path A and Path B following Scopolamine treatment. In addition to learning acquisition being impaired, memory was also adversely affected as indicated by elevated latency times and error numbers in both sexes in the memory phase of Path A following scopolamine treatment. The learning and memory data (i.e. escape latency and error counts) generated using the Biel water maze paradigm are consistent with previously reported effects of scopolamine on maze performance. Therefore, the study report for this positive control study provided sufficient information to conclude that this study was appropriately designed and conducted and demonstrated that the Biel Water Maze testing paradigm is capable and sensitive enough to measure treatment related impairments in learning and memory in male and female weanling rats.

b. Historical Control Data (i.e. for those data used to aid interpretation of study findings):

(1) * Mean Gestation Length
**** Mean Pre-weaning Body Weight**
Appendix I WIL Reproductive Historical Control Data
Sex: * Female; **Male and Female
Species: Rat
Strain: Crl:CD(SD)
Number of Studies / Control Groups: 163/221
Study Type: All
Range of Study Dates: 08/00 - 10/09

***Mean Gestation Length (days)**

Mean	SD	N	Minimum / Maximum
21.9	0.18	97	21.5 / 22.3

****Male Pre-weaning Body Weight (g)**

	Mean	SD	N	Minimum / Maximum Mean
PND 14	30.0	3.16	80	20.2 / 36.4
PND 17	38.0	2.59	32	30.7 / 42.0
PND 21	48.6	4.83	85	32.8 / 57.8

****Female Pre-weaing Body Weight (g)**

	Mean	SD	N	Minimum / Maximum Mean
PND 14	29.7	2.99	80	20.3 / 34.8
PND 17	36.6	2.40	32	30.0 / 40.3
PND 21	46.5	4.44	85	32.8 / 54.9

(2) * Locomotor Activity
Appendix M WIL Locomotor Activity Historical Control Data
Sex: Male and Female
Species: Rat
Strain: Crl:CD(SD)
Study Type: DNT, PRE-POST NATAL
Range of Study Dates: 09/30/2007 - 07/16/2010

*** Locomotor Activity (1 hour sessions)**

Sex	Age(PND)	Total Activity				Ambulatory Activity			
		N	Mean	SD	Min/Max	N	Mean	SD	Min/Max
M	13	6	**1278**	574.8	781/2386	6	**317**	209.4	123/693
M	17	6	**2791**	522.5	1877/3392	6	**1053**	241.1	614/1272
M	21	14	**2922**	1152	1636/6443	14	**1015**	468.3	419/2409
M	61	15	4538	1829.1	1874/9866	15	994	447.1	355/2249

F	13	6	**1786**	467.5	1118/2344	6	**566**	207.1	266/793
F	17	6	**2725**	752.5	1692/3921	6	**1053**	414.9	498/1729
F	21	14	**3020**	975	1708/5866	14	**1101**	428.1	457/2187
F	61	15	5149	2133	2421/12042	15	1443	635.2	571/3425

N.B.: It is generally expected that the normal developmental change in total activity counts between PND 13 and 21 should consist of an increase in activity on PND 17 (relative to PND 13) and then a decrease in activity by PND 21 (relative to PND 17), i.e. over those three days the peak activity occurs on PND 17. However, the WIL/Kinder Scientific laboratory historical locomotor data provided above (copied from Appendix M in the final report) do not reliably demonstrate the expected normal pattern of development for locomotor activity between PND 13 and 21. Instead, the historical data show a continuous increase in male total activity, female total activity and female ambulatory activity, i.e. peak activity occurring on PND 21. Male ambulatory activity did show a slight decrease in PND 21 ambulatory activity relative to PND 17. However, the same type of Kindler Scientific locomotor activity system that produced the historical data was used in the present study (MRID 48766703 and produced the locomotor data shown below ($M/F^{MRID48766703}$) for total activity (males and females combined) and for ambulatory activity (males and females combined) clearly showing the expected normal pattern of development for locomotor activity between PND 13 and 21 with activity peaking on PND 17:

		Total Activity		Ambulatory Activity	
$M/F^{MRID48766703}$	PND	mean	SD	mean	SD
	13	**2222**	1347.2	**856**	761.4
	17	**3622**	1934.6	**1549**	1036.6
	21	**2444**	703.2	**901**	278.3

Consequently, although the WIL historical data (collected using the Kinder Scientific locomotor system) do not clearly show the normal pattern of locomotor activity development, the Kinder Scientific equipment used in this study did reliably and accurately show the expected normal pattern of locomotor activity in control animals.

(3) * WIL Biel Water Maze (Female PND 22)

Appendix N WIL Manual Biel Water Maze Historical Control Data
Sex: Female
Species: Rat
Strain: Crl:CD(SD)
Study Type: DNT
Range of Study Dates: 06/14/1999 - 08/11/2010

***Path – Trial Type/No.**	**Mean**	**SD**	**# Studies**	**Min/Max**
Learning Trials Escape Times (sec)				
A1	5	16.5	30	46.6 / 110.5
A2	70.9	13.01	30	48.2 / 114.6
A3	61.9	13.36	30	40.3 / 95.2
A4	46.4	8.72	30	29.3 / 65
B5	147.4	14.93	30	115.7 / 170.9
B6	121.1	17.83	30	82.5 / 161
B7	105.9	20.83	30	63.6 / 139.4
B8	85.6	18.32	30	46.6 / 114.5
B9	79.4	20.86	30	51.9 / 125
B10	60.8	16.21	30	33.6 / 102.1

Memory Trials Escape Times				
A11	76.3	13.78	30	50.8 / 104.7
A12	56.2	11.38	30	34.2 / 82.2
Learning Trials Errors (count)				
A1	15.2	3.3	31	8 / 20.7
A2	13.6	2.64	31	6.7 / 19.1
A3	12.6	3.31	31	6.7 / 19.7
A4	9.2	2.27	31	5.8 / 14.2
B5	28.8	4.89	31	13.7 / 39
B6	23.8	4.4	31	15.8 / 35
B7	21.2	5.54	31	6 / 31
B8	16.6	4.25	31	8.4 / 24
B9	16.5	5.51	31	8.1 / 28
B10	12.2	4.57	31	5.2 / 23.9
Memory Trials Errors				
A11	20.7	5.0	30	11.4 / 31
A12	13.5	3.62	30	7 / 21.8

II. RESULTS:

A. PARENTAL ANIMALS:

1. Mortality and clinical and functional observations:

F_0 Mortality

One F_0 female (ID No. 99419) in the 100 mg/kg/day treatment group was found dead on gestation day 21. At necropsy, this female was found to have 14 dead fetuses in utero, but no notable macroscopic lesions. The cause of death for this female could not be determined from the macroscopic examination. All other F_0 females survived to the scheduled necropsy.

F_0 Post-Dosing Clinical Observations

Treatment related clinical findings were observed in all F_0 females in the 100 mg/kg/day group at approximately 15-30 minutes post-dosing generally throughout the treatment period and included behavior/CNS-related findings (sitting with the head held low, hypoactivity, rocking, lurching, or swaying while ambulating, flattened body, piloerection, tremors/convulsions, and slightly drooping eyelids), cardio-pulmonary findings (decreased respiration), and material/autonomic-related findings (yellow material on various body surfaces, clear material around the mouth/salivation, lacrimation, and dilated pupils). These findings generally continued to the 2-hour post-dosing observations, but at decreased incidences, and were not observed at the daily clinical observations prior to dose administration.

At 15 mg/kg/day, increased incidences of sitting with the head held low, hypoactivity, slightly drooping eyelids, dilated pupils, a flattened body, decreased respiration, and clear material around the mouth were observed at approximately 15-30 minutes post-dosing, but generally did not persist to the 2-hour post-dosing observations or the daily examinations the following morning. Aside from single occurrences of decreased respiration and clear material around the mouth in a limited number of animals at approximately 15-30 minutes post-dosing, no test-substance-related clinical findings were observed at 5 mg/kg/day at the daily or post-dosing observations.

Text Table 2A summarizes the post-dose clinical findings in F_0 females.

Text Table 2A. Test Substance-Related Clinical Findings Observed Following Dose Administration (*Presented as total occurrence/number of animals*)*								
Group:	**0 mg/kg/day**		**5 mg/kg/day**		**15 mg/kg/day**		**100 mg/kd/day**	
Time Post-Dosing:	**15-30 mins**	**2hrs**	**15-30 mins**	**2hrs**	**15-30 mins**	**2hrs**	**15-30 mins**	**2hrs**
Behavior/CNS								
Sitting with head held low	0/0	0/0	0/0	0/0	72/22	7/5	241/25	85/24
Hypoactivity	0/0	0/0	0/0	0/0	35/16	0/0	431/25	69/20
Rocking/lurching/swaying while ambulating	0/0	0/0	0/0	0/0	0/0	0/0	545/25	91/23
Body flattened	0/0	0/0	0/0	0/0	6/4	1/1	328/25	48/19
Piloerection	0/0	0/0	0/0	0/0	0/0	0/0	30/18	0/0
Tremors	0/0	0/0	0/0	0/0	0/0	0/0	11/9	1/1
Clonic convulsions	0/0	0/0	0/0	0/0	0/0	0/0	2/2	0/0
Material/ Autonomic								
Yellow material ventral thoracic area	0/0	0/0	0/0	0/0	0/0	0/0	3/2	12/7
Yellow material ventral abdominal area	0/0	0/0	0/0	0/0	0/0	0/0	9/6	49/19
Yellow material urogenital area	0/0	0/0	0/0	0/0	0/0	0/0	10/8	48/16
Eyelids slightly drooping	0/0	0/0	0/0	0/0	60/21	6/4	118/25	91/23
Lacrimation left eye	0/0	0/0	0/0	0/0	1/1	0/0	10/5	0/0
Lacrimation right eye	0/0	0/0	0/0	0/0	1/1	0/0	8/5	0/0
Dilated pupil left eye	0/0	0/0	0/0	0/0	13/8	0/0	337/25	70/21
Dilated pupil right eye	0/0	0/0	0/0	0/0	13/8	0/0	337/25	70/21
Clear material around mouth	0/0	0/0	0/0	0/0	12/6	0/0	214/23	8/7
Cardio-Pulmonary								
Decreased respiration	0/0	0/0	1/1	0/0	3/3	0/0	290/25	67/23
** Data obtained from page 75 in the study final report.*								

Maternal Care Observations

A decreased incidence of females in the 100 mg/kg/day group that were on the nest and a higher incidence of females in the 100 mg/kg/day group that were away from the nest and not actively eating, drinking, grooming, or tending to the litter were noted at approximately 15-30 minutes post-dosing and continued to a lesser extent in the 2-hour post-dosing observations. In addition, higher incidences of 1-3 pups and more than 3 pups outside the nest were observed for the 100 mg/kg/day group at approximately 15-30 minutes and to a lesser extent at 2 hours post-dosing. These differences in maternal care between control and the 100 mg/kg/day groups were not as notable in the daily observations prior to dosing.

Although the final report stated that "The maternal care behavior for the 5 and 15 mg/kg/day groups was similar to the control group at approximately 15-30 minutes post-dosing, 2 hours post-dosing, and at the daily examinations," it would be more accurate to note that there was a tendency for certain elements indicative of maternal care to be affected in the 5 and 15 mg/kg/day groups of F_0 females. Specifically, relative to concurrent controls, there were slightly increased incidences of F_0 females being away from the nest (not actively eating, drinking, grooming or tending to the litter) and of scattered litters with 1-3 pups outside the next at 15-30 minutes post-dosing for both the 5 and 15 mg/kg/day dose groups. These effects appeared to continue to some extent into the 2-hour post dose period mainly for the 15 mg/kg/day group. The suggested decrement in maternal care based on the observations noted at the 100 mg/kg/day dose level and the tendency for a slight effect on maternal care at the 15 mg/kg/day dose level may be consistent with the extent to which clinical observations were affected in the F_0 females at each of these dose levels. However, in the absence of any notable clinical observations or other treatment related effects in the 5 mg/kg/day group of F_0

females, the apparent slight changes in maternal behavior at this dose level are not considered biologically relevant.

Text Table 2B summarizes the post-dose clinical findings for indices related to maternal care in F_0 females.

Text Table 2B. Test Substance-Related Clinical Findings Related to Maternal Care

Following Dose Administration (Presented as total occurrence/number of animals)*

Group:	0 mg/kg/day		5 mg/kg/day		15 mg/kg/day		100 mg/kd/day	
Time Post-Dosing:	**15-30 mins**	**2hrs**	**15-30 mins**	**2hrs**	**15-30 mins**	**2hrs**	**15-30 mins**	**2hrs**
Maternal Care								
Maternal Care: On Pups	137/24	152/24	133/25	154/25	141/25	149/25	59/22	91/22
Maternal Care: Away From Nest; Actively Tending to Litter (Retrieving pups or building nest)	1/1	3/2	0/0	2/2	0/0	0/0	0/0	3/3
Maternal Care: Away From Nest; Actively Eating, Drinking or Grooming	14/12	2/2	16/12	5/4	14/13	8/6	18/12	22/14
Maternal Care: Away From Nest; Not Actively Eating, Drinking or Grooming and Not Tending to Litter (Sleeping, Resting on Food Jar)	16/13	11/8	24/15	13/9	20/14	18/15	84/23	46/20
Litter Not Scattered; All Pups in Nest	164/24	165/24	163/25	172/25	166/25	168/25	102/23	142/23
Litter Scattered; 1-3 Pups Outside Nest	2/2	3/2	9/7	1/1	8/8	7/5	47/20	17/12
Litter Scattered; More Than 3 Pups Outside Nest	1/1	0/0	1/1	1/1	1/1	0/0	12/8	3/3

** Data obtained from pages 188-189 in the final study report.*

One additional facet of maternal behavior is cannibalization of pups. A relatively greater number of pups were missing during the postnatal period in the 100 mg/kg/day dose group (20 pups from 12 litters) than in the control (3 pups from 2 litters), the 5 mg/kg/day (2 pups from 2 litters) or the 15 mg/kg/day (2 pups from 2 litters) groups. The missing pups were presumed to have been cannibalized.

F_0 Detailed Clincal Observations (Functional Observational Battery)

In the home cage observations, more F_0 females in the 100 mg/kg/day group than in the control group were observed with a flattened posture or sitting with the head held low and low or very low arousal on GD 10 and 15 and LD 10 and 20 (all differences being significant at either $p<0.05$ or $p<0.01$ and correlated with observations noted during the daily post-dosing evaluations). These effects were associated with significantly fewer females observed sitting or standing normally and having normal arousal on all test days as compared with controls (all differences being significant at $p<0.05$ or $p<0.01$). Compared with controls, fewer females in the 100 mg/kg/day group were also observed rearing in the home cage on LD 20 ($p<0.01$) and fewer females were found to be alert on observation days GD 15 and LD 20 (both $p<0.05$). One female in this treatment group exhibited slight tremor in the home cage on LD 10.

The females in the 15 mg/kg/day group exhibited no significant changes in home cage parameters on GD 10 but by GD 15 there were significantly ($p<0.05$) more females in this group as compared with the controls that were observed sitting with their heads held low,

resulting in fewer females in this group that were alert ($p<0.05$) or sitting or standing normally. On LD 10 and 20, there were still several females in the 15 mg/kg/day group that were observed sitting with their heads held low and correspondingly fewer females rated as alert but these differences were not significantly different from control. Unlike the 100 mg/kg/day group, flattened body postures were not significantly observed in the 15 mg/kg/day group of females. On lactation day 20, a small but significant ($p<0.05$) number of females in the 15 mg/kg/day group were observed rearing compared with the controls. (The investigator's final report erroneously stated that "3 of 20 females in the 15 mg/kg/day group were observed rearing compared with 11 of 20 females in the control group". In actuality there were 3 of 25 females in the 15 mg/kg/day group and 11 of 24 control females exhibiting rearing.) This reviewer does not agree with the investigator's conclusion that the decrease in rearing for the 15 mg/kg/day group was not considered treatment-related because the majority of females in that dose group were sitting or standing normally in the home cage. Since this decrease in rearing in the 15 mg/kg/day group compared with control (3 vs 11) was significant and was consistent with the even greater decrease in home cage rearing at the 100 mg/kg/day dose level, in the opinion of this reviewer the effect at the 15 mg/kg/day dose level is treatment related. No treatment related findings were observed in home cage parameters on any day of testing (i.e., GD 10 and 15 and LD 10 and 20) for the 5 mg/kg/day dose group.

With regard to the observations made during handling, significantly (all effects significant at $p<0.05$ or $p<0.01$) more females in the 100 mg/kg/day group than in the control group were observed with slight lacrimation (GD 10 and 15), slight salivation (GD 15, LD 10 and 20), slight piloerection (LD 10 and 20), slightly drooping eyelids (GD 10 and 15, LD 10), and decreased respiration (GD 10 and 15, LD 10 and 20); these effects generally correlated with observations noted during the daily post-dosing evaluations. No treatment related findings were observed in the handling observations on any day of testing (i.e., GD 10 and 15 and LD 10 and 20) for either the 5mg/kg/day or 15 mg/kg/day dose groups.

In the open field portion of the detailed clinical observations, significantly ($p<0.01$) more females in the 100 mg/kg/day group, compared with the control group, had ataxia and slightly impaired mobility on GD 10 and 15 and LD 10 and 20 (and several females with moderately impaired mobility on LD 20). A limited non-significant number of females in the 100 mg/kg/day dose group also exhibited moderately impaired mobility on LD 10, tremors (GD 10, LD 10 and 20), dragging body (LD 10) and slight to moderately coarse tremors (GD 10, LD 10 and 20). Significantly ($p<0.05$) higher mean urination counts were noted for the 100 mg/kg/day group when compared with control on GD 10 and 15 and LD 10 and 20. On lactation day 20, a significantly ($p<0.05$) higher mean backing count was observed for the 100 mg/kg/day group when compared with the control group. This reviewer does not agree with the investigator's conclusion in the final report that, "due to the small magnitude of difference (0.3 counts), this change [in backing counts] was not considered treatment-related"; in view of the various other treatment related effects on the detailed observational parameters at the 100 mg/kg/day dose level, this statistically significant small magnitude difference in open field 'backing counts' is consistent with a treatment related effect at this dose level.

At 15 mg/kg/day, no test substance-related open field observations were noted on GD 10. On GD 15 and LD 10 and 20, single occurrences of slightly impaired mobility and ataxia were observed for the 15 mg/kg/day group; although none of the differences from the control

group were statistically significant, the findings were considered related to treatment given the relationship to similar findings observed at 100 mg/kg/day. A slightly higher (significant, $p<0.05$) mean urination count was also observed for the 15 mg/kg/day group when compared with the control group on lactation day 20.

No treatment related findings were observed in the 5 mg/kg/day group for any open field parameter on GD 10 and 15 and LD 10 and 20.

No treatment related effects on pupillary response were noted for Fo females at any dose level (5, 15, and 100 mg/kg/day) on any of the detailed clinical observations days (GD 10 and 15 and LD 10 and 20).

The significant results of the detailed clinical observations for Fo females are summarized in Table 2C for the Gestation period (GD 10 and 15) and in Table 2D for the Lactation period (LD 10 and 20).

TABLE 2C. Fo Female detailed clinical observations for Gestation (only significant observations presented) [a]

Detailed Clinical Observation (Functional Observational Battery)	Dose (mg/kg/day)			
	0 (N=24)***	5 (N=25)	15 (N=25)	100 (N=24)***
Day of Observation	GD10 / GD 15	GD10 / GD 15	GD10 / GD 15	GD10 / GD 15
Home Cage				
Body Posture:				
- Sitting/Standing Normally	18 / 11	19 / 12	14 / 7	4** / 2**
- Asleep	- / 6	1 / 6	4 / 8	1 / -*
- Alert	2 / 6	1 / 5	1 / 1*	- / -*
- Sitting w Head Held Low	- / -	3 / -	4 / 7*	4 / 9**
- Flattened	- / -	- / -	- / 2	15** / 13**
Arousal:				
- Very Low	1 / -	2 / -	- / -	7 * / 7**
- Low	- / -	- / -	2 / 5	10** / 10 **
- Normal	23 / 24	23 / 25	23 / 20	7** / 7**
Handling Observations				
Lacrimation:				
- None	24 / 24	25 / 25	25 / 24	18* / 17**
- Slight	- / -	- / -	- / 1	6* / 7**
Salivation:				
- None	24 / 24	25 / 25	25 / 25	20 / 5**
- Slight	- / -	- / -	- / -	4 / 19**
Palpebral Closure				
- Eyelids Wide Open	24 /24	25 / 25	24 / 22	13** / 16**
- Slightly Drooping	- / -	- / -	1 / 3	11** / 8**
Respiratory Rate:				
- Normal (80-110)	24 / 24	25 / 25	25 / 25	17** / 14**
- Decreased (<80)	- / -	- / -	- / -	7** / 10**
Open Field Observations				
Mobility:				
- Normal	24 / 24	25 / 25	25 / 24	13** / 14**
- Slightly Impaired	- / -	- / -	- / 1	11** / 10**
Gait:				
- Normal	24 / 24	25 / 25	25 / 24	13** / 14**
- Ataxia	- / -	- / -	- / 1	11** / 10**
Urination Counts (mean ± SD)	0.8±0.68 / 0.4±0.83	0.4±0.58 / 0.5±0.65	0.8±0.72 / 0.6±0.71	1.5±0.78** / 1.8±0.79**

[a] Data, obtained from Table S4 and S5 pages 190-203 in the final report, are numbers of animals exhibiting response, unless noted otherwise.
* Statistically different ($p < 0.05$) from the control. ** Statistically different ($p < 0.01$) from the control.
*** One female (F_0) each in the 0 mg/kg/day and the 100 mg/kg/day dose groups was not gravid.

TABLE 2D. F_0 Female detailed clinical observations for Lactation (only significant observations presented) [a]

Detailed Clinical Observation (Functional Observational Battery)	Dose (mg/kg/day)			
	0 (N=24)***	5 (N=25)	15 (N=25)	100 (N=23)***
Day of Observation	LD10 / LD 20	LD10 / LD 20	LD10 / LD 20	LD10 / LD 20
Home Cage				
Body Posture:				
- Sitting/Standing Normally	17 / 8	17 / 14	19 / 13	2** / 1*
- Asleep	- / -	- / 1	- / 4	1 / 1
- Alert	3 / 5	3 / 1	1 / 1	- / -*
- Sitting w Head Held Low	1 / -	- / -	3 / 4	15** / 8*
- Flattened	- / -	- / -	1/ -	5* / 13**
- Rearing	3 / 11	5 / 10	1 / 3*	- / - **
Arousal:				
- Very Low	1 / -	- / -	- / -	3 / 6
- Low	1 / -	- / -	4 / 3	16** / 14 **
- Normal	22 / 24	25 / 25	21 / 22	4** / 3**
Handling Observations				
Lacrimation:				
- None	23 / 24	25 / 25	24 / 24	21 / 21
- Slight	1 / -	- / -	1 / 1	2 / 2
Salivation:				
- None	24 / 24	25 / 25	25 / 25	14** / 9**
- Slight	- / -	- / -	- / -	9** / 14**
Piloerection:				
- None	24 / 24	25 / 25	25 / 25	17** / 14**
- Slight	- / -	- / -	- / -	6** / 9**
Palpebral Closure				
- Eyelids Wide Open	24 /24	25 / 25	24 / 24	19 / 22
- Slightly Drooping	- / -	- / -	1 / 1	11** / 1
Respiratory Rate:				
- Normal (80-110)	23 / 24	25 / 25	25 / 25	16* / 13**
- Decreased (<80)	- / -	- / -	- / -	7** / 10**
- Increased (>110)	1 / -	- / -	- / -	- / -
Open Field Observations				
Mobility:				
- Normal	24 / 24	25 / 25	24 / 24	4** / 5**
- Slightly Impaired	- / -	- / -	1 / 1	18** / 13**
- Moderately Impaired	- / -	- / -	- / -	1 / 5**
Gait:				
- Normal	24 / 24	25 / 25	24 / 24	15** / 9**
- Body Drags	- / -	- / -	- / -	2 / -
- Ataxia	- / -	- / -	1 / 1	6** / 14**
Tremors:				
- None	24 / 24	25 / 25	25 / 25	20 / 19
- Slight (1.5 mm)	- / -	- / -	- / -	2 / 1
- Moderately Coarse (3 mm)	- / -	- / -	- / -	1 / 3
Backing Counts (mean $\pm$SD)	0 / 0	0 / 0	0 / 0	0.2$\pm$0.74 / 0.3$\pm$0.88*
Urination Counts (mean $\pm$ SD)	0.5+0.98 / 0.1$\pm$0.28	0.6$\pm$0.96 / 0.3$\pm$0.75	1.0$\pm$1.31 / 0.8$\pm$0.88*	3.7$\pm$2.05** / 2.7$\pm$1.18**

[a] Data, obtained from Table S6 on pages 204 -217 in the final report, are numbers of animals exhibiting response, unless noted otherwise.

* Statistically different ($p < 0.05$) from the control.

** Statistically different ($p < 0.01$) from the control.

*** One female (Fo) each in the 0 mg/kg/day and the 100 mg/kg/day dose groups was not gravid and one female (Fo) in the 100 mg/kg/day group died on gestation day 21.

2. Body weight and food consumption:

a. Gestation Body Weights/Weight Gains/Food Consumption

Treatment related slightly lower mean body weight gains were noted for the 100 mg/kg/day group when compared with the control group generally throughout the gestation treatment period; the differences were occasionally significant ($p<0.01$). The overall mean body weight gain for the 100 mg/kg/day group for the entire gestation treatment period (gestation days 6-20) was significantly ($p<0.01$) lower than the control group. Mean body weights for the 100 mg/kg/day group were 4.2% and 4.6% lower than the control group on gestation days 18 and 20, respectively (both significant at $p<0.05$).

Mean body weights and body weight gains during gestation were not affected at either the 5 or 15 mg/kg/day dose levels. In one instance mean body weight gain for the 5 mg/kg/day dose group was statistically lower than control ($p<0.01$) but, since this decrease was transient and was not observed in a dose-dependent manner, it was concluded that this difference did not reflect an apparent treatment related effect.

Consistent with the lower body weight gains and body weights for the 100 mg/kg/day group, treatment related decreases in mean food consumption (evaluated as g/animal/day and g/kg/day) relative to control were also noted for the 100 mg/kg/day group over the periods of GD 6-9 and 9-12 (both $p<0.01$). Significantly ($p<0.05$) lower mean food consumption (g/animal/day) was also noted for this group during gestation days 15-18, but the magnitude of difference from the control group (2 g) was small, and therefore this difference was not considered treatment-related; also, this lower mean food consumption on GD 15-18 was no longer significantly different from control when food consumption was based on body weight (g/kg body weight/day). When the entire gestation treatment period (gestation days 6-20) was evaluated, mean food consumption was significantly ($p<0.01$) lower than the control group. Mean food consumption during gestation was unaffected by either the 5 and 15 mg/kg/day dose treatments.

b. Lactation Body Weights/Weight Gains/Food Consumption:

Mean body weight gain for the 100 mg/kg/day group was significantly ($p<0.01$) lower than the control group during LD 1-4. With the exception of a significantly ($p<0.05$) lower mean body weight gain during LD 7-11, mean body weight gains for the 100 mg/kg/day group were similar to the control group for the remainder of the lactation period and when the entire lactation period (LD 1-21) was evaluated. Mean body weights for the 100 mg/kg/day group were 4.5% to 6.3% lower than the control group on each day of measurement during LD 4-17 (significant, $p<0.05$ or $p<0.01$), but were similar to the control group on LD 21.

Mean body weights and body weight gains during lactation were unaffected by test substance administration at 5 and 15 mg/kg/day. Significantly ($p<0.05$) lower mean body eights were noted for the 5 mg/kg/day group on lactation days 11 and 14. However, these differences were not observed in a dose-responsive manner and did not correlate with other signs of toxicity at this dose level. Therefore, no relationship to the test substance was apparent.
Consistent with the decreased body weights of the F_0 females in the 100 mg/kg/day during lactation, mean food consumption for this group was significantly lower than the

control group at each measurement period from LD 4-7 through LD 17-21 and for the overall lactation period (LD 1-21) (all $p<0.01$).

Mean food consumption during lactation was basically unaffected by treatment at the 5 and 15 mg/kg/day dose levels. In two measurement periods, LD 7-11 and 11-14 mean food consumption (g/animal/day) for the 5 mg/kg/day group was statistically ($p<0.05$) lower than the control group; due to the absence of a dose-response, these decrements were considered sporadic and not treatment-related. Furthermore, these apparent differences between mean food consumption for the 5 mg/kg/day group and control were no longer significant when mean food consumption was based on body weight (g/kg body weight/day).

Selected group mean body weights and food consumption values for pregnant (Gestation) and nursing (Lactation) F_0 dams are summarized in Tables 3a and 3b, respectively.

TABLE 3a. Mean (±SD) F_0 maternal body weight/gain and food consumption during Gestation

Observations/study day or interval	Dose (mg/kg/day)			
	0 (N=24)	5 (N=25)	15 (N=25)	100 (N=24)
MEAN BODY WEIGHT (g)[a] Gestation day 0	265 (11.6)	262 (15.4)	264 (14.3)	265 (14.7)
Gestation day 6	290 (13.4)	288 (16.9)	289 (15.8)	288 (11.5)
Gestation day 9	300 (12.8)	297 (16.7)	300 (19.3)	293 (11.7)
Gestation day 12	317 (13.6)	312 (17.8)	315 (21)	309 (12.5)
Gestation day 15	336 (15.7)	328 (18.1)	334 (20.7)	326 (13.3)
Gestation day 18	378 (20.1)	369 (21.5)	376 (23.2)	362 (16.4)* [-4.2%][e]
Gestation day 20	412 (23.8)	401 (24.4)	409 (24.6)	393 (19.8)* [-4.6%][e]
*p<0.05 [a] Data obtained from Table S7 on pp. 218-219 of the final report				
MEAN BODY WEIGHT CHANGES (g) [b] Gestation days 0-6	25 (6.4)	26 (6.2)	25 (6.8)	24 (6.7)
Gestation days 6-9	9 (4.6)	9 (4)	11 (5.3)	5 (4.1)**
Gestation days 9-12	17 (4)	15 (3.3)	15 (4.9)	16 (5.4)
Gestation days 12-15	20 (4.6)	16 (3.9)**	19 (4.2)	17 (5.1)
Gestation days 15-18	42 (6.5)	41 (6.3)	42 (5.2)	36 (6.8)**
Gestation days 18-20	34 (5.5)	32 (6.4)	33 (7.4)	31 (6.5)
Gestation days 6-20	122 (15.8)	113 (13.3)	120 (12.6)	104 (14.9)** [-15%][e]
* p<0.05; **p<0.01 [b] Data obtained from Table S8on pp. 220-221 of the final report				
MEAN FOOD CONSUMPTION (g/animal/day) [c] Gestation days 0-6	21 (1.2)	20 (1.8)	21 (1.9)	21 (1.3)
Gestation days 6-9	21 (1.8)	21 (1.7)	21 (2.8)	18 (1.9)** [-14%]
Gestation days 9-12	23 (2.3)	23 (2.3)	23 (2.4)	20 (2.1)** [-13%]
Gestation days 12-15	23 (2.2)	22 (1.9)	24 (2.1)	23 (1.8)
Gestation days 15-18	26 (2.2)	26 (2.5)	26 (2.6)	`24 (1.7)* [-7.2]
Gestation days 18-20	26 (2.3)	26 (2.4)	26 (2.1)	26 (2.7)
Gestation days 16-20	24 (1.9)	23 (1.9	24 (2.1)	22 (1.6)** [-8.3%]
MEAN FOOD CONSUMPTION (g/kg body weight/day)[d] Gestation days 6-20	70 (4.5)	70 (4.3)	71 (3.2)	66 (4.1)* [-5.7%]

* p<0.05; **p<0.01

[c] Data obtained from Table S11 on pp. 226-227 of the final report

[d] Data obtained from Table S12 on pp. 229 of the final report

[e] Numbers in brackets equal percent different from controls; calculated by Reviewer.

TABLE 3b. Mean (±SD) F_0 maternal body weight/gain and food consumption during Lactation

Observations/study day or interval	Dose (mg/kg/day)			
	0 (N=24)	5 (N=25)	15 (N=25)	100 (N=23)***
MEAN BODY WEIGHT (g)[a] Lactation day 1	311 (16.4)	302 (18)	309 (22.5)	302 (14.8)
Lactation day 4	323 (16.3)	311 (20.8)	319 (24.6)	306 (16.6)* [-5.2][e]
Lactation day 7	330 (13.5)	319 (17.7)	326 (25.8)	315 (16.1)* [-4.5]
Lactation day 11	350 (17.7)	334 (21.2)*	339 (27.4)	328 (16)** [-6.3]
Lactation day 14	357 (18.8)	341 (19.9)*	349 (25.5)	336 (19.1)** [-5.9]
Lactation day 17	362 (20.9)	350 (21)	352 (24.4)	342 (15.9)** [-5.5]
Lactation day 21	344 (19.7)	336 (19)	338 (21.9)	334 (20.7)
*p<0.05; ** p<0.01 *** One Fo dam in 100 mg/kg/day group died on GD 21 [a] Data obtained from Table S9 on pp. 222-223 of the final report				
MEAN BODY WEIGHT CHANGES (g) [b] Lactation days 1-4	12 (7.2)	9 (7.2)	10 (5.6)	4 (8.1)** [-67%]
Lactation days 4-7	7 (8)	8 (6.8)	7 (8.5)	9 (6.7)
Lactation days 7-11	19 (11.1)	15 (7.6)	14 (8.1)	13 (7.3)* [-32%]
Lactation days 11-14	8 (9.1)	7 (9.1)	9 (7.4)	8 (7.7)
Lactation days 14-17	4 (13.3)	8 (10.5)	3 (8.8)	6 (9.1)
Lactation days 17-21	- 18 (16.1)	- 14 (14.6)	- 14 (14.5)	- 8 (11.9)
Lactation days 1-21	33 (15.7)	34 (10.9)	29 (12.9)	32 (11.2)
* p<0.05; **p<0.01 [b] Data obtained from Table S10 on p. 224-225 of the final report				
MEAN FOOD CONSUMPTION (g/animal/day) [c] Lactation days 1-4	36 (4.6)	37 (8.2)	37 (5.3)	34 (6.1)
Lactation days 4-7	43 (4.3)	43 (6.4)	43 (5.5)	38 (2.8)** [-12%]
Lactation days 7-11	56 (3.9)	52 (6.4)*	54 (6.2)	47 (6.3)** [-16%]
Lactation days 11-14	65 (5.7)	60 (5.3)*	63 (5)	56 (8.6)** [-14%]
Lactation days 14-17	69 (5.5)	67 (5.4)	69 (5.9)	62 (8.5)** [-10%]
Lactation days 17-21	75 (6.4)	73 (6.7)	75 (6.9)	65 (9.4)** [-13%]
Lactation days 1-21	58 (4)	56 (4.5)	57 (5.1)	51 (6.3)** [-12%]
MEAN FOOD CONSUMPTION (g/kg body weight/day)[d] Lactation days 1-21	171 (12.3)	171 (12.7)	172 (12.1)	157 (18.2)** [-8.2%]

* p<0.05; **p<0.01

[c] Data obtained from Table S13 on pp. 230-231 of the final report

[d] Data obtained from Table S14 on p. 233 of the final report

[e] Numbers in brackets equal percent different from controls; calculated by Reviewer.

3. <u>Test substance intake</u>: Not applicable to the oral gavage dosing used in this study.

4. <u>Reproductive performance</u>:

Reproductive performance is summarized in Table 4. The investigator's final report states that "No test substance-related effects were noted on mean gestation lengths or the process of parturition at any dosage level. Mean F_0 gestation lengths in the test substance-treated groups

were similar to the control group value. The mean gestation lengths in the 5, 15, and 100 mg/kg/day groups were 21.7, 21.9, and 22.2 days, respectively, compared to mean gestation lengths of 21.8 days in the concurrent control group and 21.9 days in the WIL historical control data (see page 27 of this document). According to the study author, although the difference between the control and 100 mg/kg/day group was significant ($p<0.05$), a difference of 0.4 days is not considered toxicologically significant." This reviewer agrees that there did not appear to be any significant treatment related effects on the process of parturition at any dose level, and that there were no treatment related effects on gestation length at the 5 and 15 mg/kg/day dose levels. However, this reviewer does not agree that there were no treatment related effects on mean gestation lengths at the 100 mg/kg/day dose level. The difference in mean gestation length between the control (21.8 days) and the 100 mg/kg/day dose group (22.2 days) is significant ($p<0.05$) and, although small in magnitude, does appear consistent with other significant effects of at the 100 mg/kg/day dose level at various times during the gestational period, including decreased body weights and body weight gains, decreased food consumption, and a variety of post-dose clinical observations and detailed clinical observations. Consequently, the small but significant increase in gestation length for the 100 mg/kg/day dose group is considered a treatment related effect. No signs of dystocia were noted at any dosage level.

TABLE 4. Reproductive performance [a]

Observation	Dose (mg/kg/day)			
	0	**5**	**15**	**100**
Number mated F_0 females on study	25	25	25	25
No predelivery findings (# observations/# litters)	105/25	97/25	101/25	118/25
Number of litters	24 [b]	25	25	23 [b]
Intercurrent deaths	0	0	0	1 [b]
Mean (±SD) gestation duration (days)	21.8 (0.51)	21.7 (0.56)	21.9 (0.33)	22.2 (0.39)*
No significant findings during parturition (# observations/# litters)	31/24	31/25	33/25	29/23
Incidence of dystocia	0	0	0	0

[a] Data obtained from page 82, Table S1 on p 181, and Tables S15-16 on pp 234-235 in the study final report.

[b] In the 0 and 100 mg/kg/day group 1 F_0 female each was non-gravid and in the 100 mg/kg/day group 1 F_0 female died on GD 21 with 14 dead fetuses *in utero*.

* Statistically different from control, $p<0.05$.

5. **Maternal postmortem results (Fo Macroscopic Examinations)**: One F_0 female in the 100 mg/kg/day group was found dead on gestation day 21. At necropsy, this female had 14 dead fetuses in utero, but no notable macroscopic lesions. All other females survived to the scheduled necropsies. One F_0 female in the control group and 1 F_0 female in the 100 mg/kg/day group failed to deliver and were determined to be nongravid. No internal lesions were observed for these females. At the lactation day 21 necropsy, no treatment related internal findings were observed. Also, no significant treatment related effects were observed on the number of former implantation sites and the number of unaccounted-for sites.

B. OFFSPRING:

1. Viability and clinical signs:

The mean number of pups born per litter and the mean live litter size (PND 0) were slightly lower in the 100 mg/kg/day group (neither statistically significant) than the control group. Relative to the number of pups born, the mean postnatal survival (% per litter) for the 100 mg/kg/day group was notably lower than the control on PND 4 (pre-culling), but this decrease was also not statistically significant. The absence of any notable differences in the mean percentage of males per litter at birth between the control and 100 mg/kg/day groups indicates the sex ratios were unaffected by treatment. In the Pathology Report (Appendix F, page 9) for this study (page 563 of the final report) it is stated that the lower pre-weaning pup survival in the 100 mg/kg/day group was considered treatment related, and the reviewer agrees with this conclusion. The mean number of pups born, live litter size (PND 0), percentage of males per litter at birth, and postnatal survival were relatively unaffected at the 5 and 15 mg/kg/day doses administered to the Fo maternal animals.

Litter size and viability *(survival)* results from pups during lactation are summarized from the study final report in Table 5 below. The study report did not present mean litter size values across the postnatal period, live birth index, viability index, or lactation index.

TABLE 5. Litter size, viability and notable clinical findings [a]

Observations of Litter size and viability	Dose (mg/kg/day)			
	0 (N=24)	5 (N=25)	15 (N=25)	100 (N=23)
Mean Number Pups Born Per Litter (SD)	15.2 (2.13)	14.7 (2.41)	15.1 (2.04)	14.5 (2.45)
Mean Number Live Pups on PND 0 (SD)	15.1 (2.15)	14.6 (2.43)	14.9 (2.04)	14.0 (2.62)
Mean % Males Per Litter on PND 0 (SD)	48.7 (13.34)	50.6 (11.34)	49.1 (13.13)	53.8 (13.92)
Mean % Pups Per Litter (SD): PND 4 (Pre-culling) Relative to Number Born	96.2 (4.15)	97.7 (4.70)	96.9 (4.99)	87.7 (17.32)
Mean % Pups Per Litter (SD): PND 21 Relative to PND 4 (Post-culling)	100 (0)	99.4 (2.86)	99.5 (2.50)	97.6 (6.51)
* Statistically different from control, p<0.05 ** Statistically different from control, p<0.01				
Notable Clinical Findings for Pups PND 1 – PND 21				
Found Dead (number pups/number litters)	11/9	7/5	11/8	24/12
Missing [presumed cannibalized] (number pups/number litters	3/3	2.2	2.2	20/12

[a] Data obtained from Tables S21, S22 and S23 on pp. 240-245 in the study final report.

As presented in Table 5 above, the only *notable clinical findings* for the F_1 offspring over the period of PND 1 to PND 21 were that considerably more pups (litters) were found dead (24 pups in 12 litters) or missing and presumed cannibalized (20 pups in 12 litters) in the 100 mg/kg/day treatment group than were found in the control group (11 pups in 9 litters and 3 pups in 3 litters, respectively). This underscores the treatment related reduced litter sizes at the 100 mg/kg/day dose level, which mean data are also presented in Table 5 and discussed above. The other measures of general physical condition (defined as the occurrence and severity of general clinical findings) of surviving F_1 pups from PND 1 to PND 21 were not remarkably affected by maternal test substance administration. The statement in the final

report (page 83) that "The general physical condition (defined as the occurrence and severity of clinical findings) of all F_1 pups in this study was unaffected by maternal test substance administration" is in the opinion of this reviewer inaccurate, since as noted above there was a treatment related increase in pup deaths over the postnatal period of PND 1 to PND 21 in the 100 mg/kg/day dose group and, as noted in a subsequent section, preweaning body weights/weight gains of F_1 pups were also affected at the 100 mg/kg/day dose level. A more accurate statement would be that, based on the occurrence and severity of the general clinical findings and not including preweaning body weight/weight gain, the general physical condition of surviving F_1 pups was unaffected by test substance administration to the F_0 maternal animals.

2. F_1 Offspring Body Weight/Weight Gain:

a. Preweaning:

Preweaning F_1 Offspring Body Weight/Weight Gain

The mean F_1 male and female pup birth weights (PND 1) were significantly ($p<0.01$) lower (8.3% and 8.8%, respectively) in the 100 mg/kg/day group compared to the control group. Mean pup body weights for both sexes in the 100 mg/kg/day group continued to be significantly lower than control on each measurement day from PND 4 to PND 21 (all $p<0.01$); at times over this postnatal period the body weights were up to 18.5% (males) and 18.3% (females) lower than control. In addition, mean pup body weight gains in the 100 mg/kg/day group males and females were lower than the control on each measurement interval throughout the postnatal period, significantly ($p<0.05$ or $p<0.01$) different from control on most of those intervals.

Mean pup body weights and body weight changes in 15 mg/kg/day group males and females during the pre-weaning period were unaffected by test substance administration to the Fo maternal animals.

Mean pup body weights for both sexes of F_1 offspring in the 5 mg/kg/day dose group were found to be 5% to 8% lower than the control group on PND 14, 17, and 21 but significantly only for males ($p<0.05$ to $p<0.01$). The mean pup body weight gains for both sexes in this dose group were also lower than the control group on the measurement intervals PND 11-14 (significant for males at $p<0.01$ and females at $p<0.05$) and PND 14-17 (significant for males only at $P<0.05$). However, since males and females in the 15 mg/kg/day dose group did not have any decreased body weights or body weight gains throughout this pre-weaning period, indicating the absence of a clear dose-response, and since the mean body weights on PND 14, 17, and 21 for the 5 mg/kg/day group were similar to the mean values in the WIL historical control database and the control group means were 10% to 11% greater than the historical control mean (see page 40 in this document), these decrements at the 5 mg/kg/day dose level were not considered treatment related.

Selected mean preweaning pup body weight data are presented in the following Table 6a and mean preweaning pup body weight gain data in Table 6b.

TABLE 6a. Mean (±SD) pre-weaning pup body weights (g) [a]

Postnatal day	Dose (mg/kg/day)							
	0 (N=24)[c]	5 (N=25)	15 (N=25)	100 (N=23)	0 (N=24)	5 (N=25)	15 (N=25)	100 (N=23)
	Males				Females			
	[Mean Pup Body Weight, grams (SD)]							
PND 1	7.2 (6.8)	7.2 (0.75)	7.3 (0.43)	6.6 (0.76)**	6.8 (0.62)	6.9 (0.70)	7.0 (0.36)	6.2 (0.79)**
PND 4 [b]	10.2 (1.17)	9.9 (1.36)	10.1 (1.21)	8.8 (1.47)**	9.6 (1.18)	9.4 (1.23)	9.6 (1.12)	8.3 (1.38)**
PND 7	16.2 (1.96)	15.5 (2.34)	16.5 (1.86)	13.2 (2.52)**	15.3 (2.13)	14.8 (2.20)	15.5 (1.86)	12.5 (2.36)**
PND 11	25.8 (2.83)	24.5 (3.10)	25.9 (2.54)	21.2 (2.99)**	24.4 (3.10)	23.5 (3.10)	24.5 (2.65)	20.1 (3.06)**
PND 14	33.5 (3.34)	31.4 (3.27)*	33.4 (3.01)	28.2 (2.83)**	31.8 (3.70)	30.2 (3.54)	31.9 (3.09)	26.9 (3.41)**
PND 17	41.2 (3.78)	37.9 (4.16)**	40.7 (3.38)	34.9 (2.94)**	39.2 (4.06)	36.7 (4.36)	39.0 (3.37)	33.4 (3.39)**
PND 21	54.2 (5.66)	49.9 (7.03)*	54.5 (4.93)	45.6 (5.31)	51.3 (6.15)	48.0 (6.80)	51.8 (4.93)	43.6 (5.53)**

[a] Data obtained from Table S24 on pages 246 – 249 in the study report.

[b] Before standardization (culling).

c Litter is experimental unit

* Statistically different from control, p<0.05
** Statistically different from control, p<0.01

TABLE 6b. Mean (±SD) pre-weaning pup body weight gain (g) [a]

Postnatal Day Interval	Dose (mg/kg/day)							
	0 (N=24)[b]	5 (N=25)	15 (N=25)	100 (N=23)	0 (N=24)	5 (N=25)	15 (N=25)	100 (N=23)
	Males				Females			
	[Mean Pup Body Weight Gain, grams (SD)]							
PND 1 - 4	2.9 (0.68)	2.7 (0.83)	2.8 (0.92)	2.3 (1.04)	2.8 (0.76)	2.5 (0.77)	2.7 (0.90)	2.0 (0.88)**
PND 4 - 7	6.0 (0.96)	5.7 (1.10)	6.4 (0.75)	4.4 (1.23)**	5.6 (1.01)	5.5 (1.04)	6.0 (0.86)	4.1 (1.26)**
PND 7 – 11	9.6 (1.13)	9.0 (1.17)	9.4 (1.04)	7.9 (1.06)**	9.1 (1.21)	8.7 (1.21)	9.0 (1.12)	7.7 (1.06)**
PND 11–14	7.8 (0.92)	6.8 (0.92)**	7.4 (0.77)	7.1 (1.20)	7.5 (1.02)	6.7 (1.01)*	7.4 (1.14)	6.7 (1.14)*
PND 14-17	7.7 (1.02)	6.6 (1.83)*	7.4 (1.14)	6.7 (1.24)*	7.3 (0.97)	6.5 (1.71)	7.1 (1.02)	6.5 (1.15)
PND 17-21	13.0 (2.35)	12.0 (3.63)	13.8 (2.20)	10.7 (3.17)*	12.2 (2.48)	11.3 (3.27)	12.9 (2.16)	10.2 (2.96)*

[a] Data obtained from Table S25 on pages 250 – 252 in the study report.

b Litter is experimental unit

* Statistically different from control, p<0.05
** Statistically different from control, p<0.01

b. Postweaning:

Postweaning F_1 Offspring Body Weight/Weight Gain (Subsets A and B F_1 Offspring)
Mean body weights for the 100 mg/kg/day group males were significantly lower than the control group on each day of measurement (P<0.01) throughout the postweaning period (PND 28-72); body weight differences ranged from 9.5% to 13.7% lower than control. Mean body weights for the 100 mg/kg/day group females were also significantly (p<0.05 to p<0.01) lower than the control group from PND 28 to PND 56 but were similar to controls for the remainder of the post-weaning period to PND 72; the significant body weight differences ranged from 7.5% to 12.8% lower than control. Decreased mean body weight gains were also noted for the 100 mg/kg/day group of males, compared with the

control group, generally throughout the entire post-weaning period but significantly decreased during PND 28-35, PND 38-42 and PND 42-49 (each $p<0.01$); mean body weight gain for this group of F_1 male offspring was also significantly ($p<0.01$) lower than control when evaluated for the overall post-weaning period (PND 28-72). The 100 mg/kg/day group of F_1 females exhibited a significantly ($p<0.05$) lower mean body weight gain, but only during PND 28-35; mean weight gains for the remainder of the post-weaning periods, as well as the overall post-weaning period (PND 28-72) were similar to controls.

Mean post-weaning offspring body weights and body weight gains in the 5 and 15 mg/kg/day dose groups were not affected by administration of the test substance to the F_0 maternal animals. Selected mean post-weaning offspring body weight data are presented in Table 7.

TABLE 7. Mean (±SD) post-weaning pup body weights and overall body weight gain (g) [a]

Postnatal day	Dose (mg/kg/day)							
	0 (N=20)	5 (N=20)	15 (N=20)	100 (N=20)	0 (N=20)	5 (N=20)	15 (N=20)	100 (N=20)
	Males				Females			
[Mean Pup Body Weight, grams (SD)]								
PND 28	95 (8.5)	91 (10.3)	100 (7.8)	82 (9.5)**	86 (8.3)	86 (8.9)	91 (5.7)	75 (9.1)**
PND 35	156 (13.7)	151 (14.4)	163 (11.8)	136 (15.6)**	134 (11.2)	134 (11.2)	140 (9.4)	119 (12.4)**
PND 42	227 (18.2)	222 (18.1)	236 (14.8)	198 (21.6)**	177 (14.9)	177 (14.0)	184 (10.8)	159 (14.8)**
PND 49	289 (20.1)	280 (21.2)	296 (20.4)	254 (25.7)**	205 (15.5)	204 (17.1)	211 (12.8)	185 (15.9)**
PND 56	353 (23.8)	342 (24)	358 (25)	313 (28)**	227 (18.4)	232 (22.6)	231 (17.5)	210 (23.4)**
PND 63	400 (28.3)	390 (26.9)	409 (29.5)	362 (32.3)**	244 (20.8)	254 (24.3)	249 (18.4)	228 (25.4)
PND 72	445 (32.7)	432 (38.9)	452 (39.1)	396 (37.4)**	264 (22.2)	273 (23)	269 (22)	251 (25.7)
[Overall Mean Pup Body Weight Gain, grams (SD)]								
PND 28 - 72	352 (28.2)	342 (30.9)	353 (34.4)	315 (28.8)**	178 (18.6)	187 (22)	180 (18.9)	177 (22.5)

[a] Data obtained from Tables S30, S31, S32 and S33 on pages 358 – 365 in the study report.
* Statistically different from control, $p<0.05$
** Statistically different from control, $p<0.01$

3. Developmental landmarks:

a. Sexual maturation (Subsets A and B F_1 offspring):

A significant ($p<0.01$) treatment related delay in the mean age of attainment of balanopreputial separation was noted for the 100 mg/kg/day group males. The attainment of balanopreputial separation in the 5 and 15 mg/kg/day groups of males was unaffected by treatment. Mean body weights for all dose groups at the age of attainment were similar to the control group.

Mean ages of attainment of vaginal patency were unaffected by any dose of the test substance administered to the F_0 maternal animals. A significant ($p<0.01$) treatment related decrease in mean body weight was noted for the 100 mg/kg/day group females as compared with controls on the day of attainment of vaginal patency.

One unexpected entry in the final report's Table S35 (page 267) which presents the mean data for vaginal patency is that N=21 is entered for the 5 mg/kg/day dose group, while all other groups have the expected N=20 (this same issue arises also in the PND 22 Beal water maze data). This may have been a typographical error but no explanation is offered in the text of the final report document.

The sexual maturation data are presented in Table 8.

TABLE 8. Mean (±SD) age of sexual maturation (days) [a]

Parameter	Dose (mg/kg/day)			
	0 (N=20)	5 (N=21)[b]	15 (N=20)	100 (N=20)
Preputial Separation (males)	44.3 (1.92) [N=20]	44.1 (1.79) [N=20]	43.3 (1.33) [N=20]	47.0 (3.59)** [N=20]
Vaginal Patency (females)	32.7 (0.87) [N=20]	32.7 (0.95) [N=21][b]	32.2 (0.97) [N=20]	32.8 (1.41) [N=20]

[a] Data obtained from S34 and S35 on pages 266-267 in the study report.

[b] See note in text above regarding this unexpected value for N.

* Statistically different from control, $p<0.05$

** Statistically different from control, $p<0.01$

b. Physical landmarks: N/A

c. Clinical Findings for Offspring After Weaning (Subsets A and B F_1 offspring):
All F_1 animals in the control, 5, 15, and 100 mg/kg/day dose groups in Subset A and B survived to their scheduled necropsies. No remarkable treatment related clinical findings were noted during the weekly examinations. Findings noted in the test substance treated groups, including hair loss and scabbing on various body surfaces, occurred infrequently, at similar frequencies in the control group, and/or in a manner that was not dose-related.

4. Behavioral assessments:

a. Functional Observational Battery (Subset A F_1 offspring):

1) Home Cage Observations:

There were no remarkable treatment related effects on home cage parameters when F_1 male and female offspring were evaluated on PND 4, 11, 21, 35, 45, and 60. On PND 21, only 1 female in the 100 mg/kg/day group was reported as being 'alert' in the home cage compared with 7 females in the control group; this difference was statistically significant ($p<0.05$). For the males, 1 male in the 100 mg/kg/day group was also reported as being 'alert' compared with 6 controls, but this difference was not statistically significant. However, the majority of females and males in the 100 mg/kg/day group were reported 'sitting/standing normally' in the home cage. Both parameters (i.e., 'sitting/standing normally' and 'alert') are considered normal behaviors for rats in the home cage. Therefore, these differences for both the females and males were not considered treatment related. On PND 45, significantly ($p<0.05$) more males in the 100 mg/kg/day group than in the control group were sitting or

standing normally in the home cage; this was not considered a toxicologically significant difference, as the behavior displayed at 100 mg/kg/day is considered normal.

2) Handling Observations:

There were no treatment related effects on handling parameters when F_1 male and female offspring were evaluated on PND 4, 11, 21, 35, 45, and 60. Findings in the 5, 15, and 100 mg/kg/day groups were noted infrequently, similarly in the control group, and/or in a manner that was not dose-related.

3) Open Field Observations:

There were no treatment related effects on open field parameters when F_1 male and female offspring were evaluated on PND 4, 11, 21, 35, 45, and 60. Findings in the 5, 15, and 100 mg/kg/day groups were noted infrequently, similarly in the control group, and/or in a manner that was not dose-related.

4) Sensory Observations:

There were no treatment related effects on sensory parameters (i.e., pupillary response) when F_1 offspring were evaluated on PND 21, 35, 45, and 60. [Note that the description of results in the final report, page 88, mistakenly included PND 4 and 11 as days on which sensory observations were made, although no pupil response data were presented in the final report Table S36 for those days. According the experimental procedures (page 54 of final report), pupil response was one of the parameters not recorded for pups on PND 4 and 11 due to their early stage of development at those times.] Findings in the 5, 15, and 100 mg/kg/day groups were noted infrequently, similarly in the control group, and/or in a manner that was not dose-related.

5) Grip Strength:

Mean forelimb grip strength measurements for the 100 mg/kg/day group males and females were significantly lower than the control group on PND 21 and 35 ($p<0.05$ for males and $p<0.01$ for females on both days). Male and female hindlimb grip strength in this same dose group was also lower than controls on both days but not at a statistically significant level. On PND 45 and 60, there were no significant treatment effects in male and female forelimb and hindlimb grip strength at the 100 mg/kg/day dose level, although on PND 45 the mean forelimb grip strength for males at this dose level was non-significantly lower than control.

At the 5 and 15 mg/kg/day dose levels, there were no significant decrements in forelimb and hindlimb grip strength for either sex, compared to the controls, at any age of evaluation. However, one isolated finding on PND 45 of a significantly ($p<0.05$) greater hindlimb grip strength was noted for the 15 mg/kg/day group females when compared with the control group. Because this increase was not observed in a dose-responsive manner, it was not considered treatment related.

Note that details of the procedure (i.e., equipment, manner of handling, number of trials, etc.) used to measure grip strength were not presented in the Procedures section of the final report. Since the final report (page 90) stated that "Positive Control Data" for grip strength were available in Appendix J, attempts were made to obtain some relevant procedural information from that source. Unfortunately, the only control data available in Appendix J for grip strength were "Historical Control Data" with no discussion of procedure and no "Positive Control Data" were available.

The grip strength data are presented in Table 9 below.

TABLE 9. Mean (±SD) grip strength in grams (Subset A Offspring) [a]

Postnatal day	Dose (mg/kg/day)							
	0 (N=20)	5 (N=20)	15 (N=20)	100 (N=20)	0 (N=20)	5 (N=20)	15 (N=20)	100 (N=20)
	Males				Females			
Forelimb								
PND 21	156.2 (31.45)	246.4 (28.35)	171.9 (27.12)	133.7 (27.89)*	159.1 (42.26)	173.5 (32.90)	167.9 (31.78)	121.5 (27.87)**
PND 35	348.6 (83.78)	336.9 (85.58)	380.3 (64.53)	291.1 (61.25)*	351.6 (60.09)	345.6 (70.19)	329.3 (64.79)	270.6 (63.31)**
PND 45	650.3 (188.46) [b]	653.0 (224.08)	660.1 (241.64)	571.8 (177.53)	540.6 (201.92)	571.2 (185.86)	623.8 (152.94)	500.0 (183.28)
PND 60	1091.3 (193.48)	1082.1 (207.67)	1120 (172.97)	1013.6 (165.79)	974.7 (150.56)	956.2 (148.87)	1001 (194.55)	896.1 (200.87)
Hindlimb								
PND 21	74.7 (18.42)	72.8 (17.92)	84.5 (19.71)	62.5 (19.72)	69.5 (18.33)	69.8 (23.42)	66.8 (17.07)	55.3 (15.43)
PND 35	159.1 (39.04)	171.6 (34.98)	166.7 (41.05)	142.9 (30.98)	162.2 (24.70)	163.5 (23.86)	165.6 (38.47)	145.4 (37.42)
PND 45	235.2 (45.99) [b]	208.3 (41.14)	234.8 (48.34)	213.7 (50.94)	222.3 (38.50)	221.8 (40.95)	255.3 (50.80)*	206.8 (40.07)
PND 60	271.0 (48.48)	280.8 (69.94)	256.7 (41.37)	282.1 (52.27)	263.7 (51.45)	275.4 (82.16)	285.1 (55.98)	240.2 (40.97)

[a] Data obtained from Table S37 on pages 332-338 in the study report.

[b] N=19

* Statistically different from control, $p<0.05$

** Statistically different from control, $p<0.01$

b. Motor Activity:

The analyses below of activity data across sessions PND 13, 17, 21 focus on determining whether there is an effect of treatment on the ontogenetic pattern of cumulative motor activity across these session. The analyses in the subsequent section below of activity data within session (PND 13, 17, 21, and 61) focus on determining the effect of treatment compared to control for cumulative activity for a session effects of the test, and also evaluating whether there was a treatment effect on the pattern of habituation within a session. It should be noted that a calculated quantitative index of the rate of habituation was not presented in any of the motor activity datasets; the effect of treatment on motor habituation was determined statistically based on the analysis of treatment by intra-session time interactions.

1) Across Session Analyses (PND 13, 17, and 21):

There were no statistically significant interactions between treatment and sex (treatment by sex or treatment by sex by session interactions) for cumulative total and ambulatory activities. Therefore, subsequent analysis on the treatment by session interaction was conducted on the pooled sexes (P; males and females combined). The overall treatment by session interactions were significant for both total (p=0.011) and ambulatory (p=0.004) activity, indicating a treatment related change in the developmental pattern of activity across these sessions. Post-hoc pairwise comparisons found that the only statistically significant treatment by combined sessions difference from the control was for the combined sessions at the 5 mg/kg/day dose level for ambulatory activity (p=0.013); although not specifically mentioned in the final report, there were no significant treatment by combined sessions difference on total activity at any dose level and no significant effects on total or ambulatory activity at 15 or 100 mg/kg/day. Based on their inspection of the data, the investigators then concluded that the difference in ontogeny of activity (investigators do not specify whether total or ambulatory) appears to be primarily due to the greater increase in activity from PND 13 to PND 17 in the 5 mg/kg/day dose group compared with the control group. This was apparently based on the significant change in ambulatory activity for sessions at the 5 mg/kg/day dose level.

However, this conclusion does not account for the absence of any significant treatment effect for total activity in sessions. To address the significant overall treatment by session interactions that were found for both total and ambulatory activities, the reviewer does not consider it adequate to attribute the difference in ontogeny of activity solely to a greater increase in activity from PND 13 to PND 15 in the 5 mg/kg/day dose group compared with the controls. Rather, based on inspection of the activity data, the reviewer considers it more appropriate at this point to suggest that the difference in ontogeny of activity (considering both total and ambulatory activities) may involve either a greater increase in activity from PND 13 to PND 17 in the 5 mg/kg/day dose group or the *lack* of a decrease in activity from PND 17 to PND 21 in the 100 mg/kg/day dose group, as compared with control. To examine the biological significance of this particular aspect of the statistical change in the pattern of ontogeny more closely, the data for females and males for total and ambulatory activity were examined separately in order to compare with historical control data, which is tabulated by sex. However, as noted on pages 28-29 of this DER document were the WIL historical control data provided in Appendix I of the study final report was found not to reliably describe the expected normal ontogenetic pattern of motor activity development across PND 13-21. The apparent increase in activity for the 5 mg/kg/day dose group on PND 17 was due to 1 male out of the group of 20 with unusually high counts (17,840 total activity counts and 10,168 ambulatory activity counts; >3 standard deviations of the mean for this group) and 1 female out of 20 with 10,608 total activity counts and 5,077 ambulatory activity counts (>2 standard deviations of the mean for this group) . When the data for this outlier male and outlier female were removed from calculations, the resulting group mean values for total and ambulatory activities, calculated for each sex, for the 5 mg/kg/day group were similar to the comparable control groups (generally within 1 standard deviation of the concurrent control group mean and the mean historical control data as copied on page 28 of this DER document).

The investigators concluded that absence of a significant treatment effect on PND 17 at the 5 mg/kg/day dose level indicates that the significant (p=0.011) treatment by session interaction was not a treatment-related effect, but instead caused by outlier pups. The reviewer does not agree with this conclusion as stated, since the absence of a significant effect on either total or ambulatory combined sessions activity for the 5 mg/kg/day group does not necessarily negate the overall treatment by session interaction which was significant for total (p=0.011) and ambulatory activity (p=0.004). Rather, in the opinion of this reviewer, the statement should be that the data merely show that the increased total (non-significant) and ambulatory (significant) combined sessions activities for the 5 mg/kg/day group were not treatment related effects. Further, as noted below, the significant findings of overall treatment by session interactions for both total activity (p=0.011) and ambulatory activity (p=0.004) may in fact involve between-session differences in activity for the 100 mg/kg/day dose group.

A visual inspection of the data indicates that at the 100 mg/kg/day dose level, but not at the 5 or 15 mg/kg/day dose level, a notable difference in ontogeny of activity (both total and ambulatory) may be particularly associated with a change in activity from PND 17 to PND 21 such that PND 17 activity was either lower than or equivalent to PND 21 activity in the 100 mg/kg/day group compared with control (the expected normal ontogenetic pattern of activity, as is indicated by the control group data, is such that PND 17 activity is notably greater than either PND 13 or PND 21). This type of abnormal pattern of locomotor development may be consistent with a generalized delay in development as evidenced by significant F_1 male and female body weight decreases at the 100 mg/kg/day dose level.

A summary of the mean total and ambulatory activity data (with male and female data pooled) for sessions PND 13, 17, and 21 is presented in Table 10a.

TABLE 10a. Cumulative total and ambulatory activity data (pooled sexes) for sessions on PND 13, 17, 21 (analyzed across sessions). [a]

Test Day	Dose (mg/kg/day)			
	0 (N[Litter]=20)	5 (N[Litter]=20)	15 (N[Litter]=20)	100 (N[Litter]=20)
Total Activity, cumulative counts (SD) ***[+ or – indicating direction of change from value in previous session]***				
PND 13	2222 (1347.2)	1622 (910.6)	1593 (1322.1)	1616 (1051.1)
PND 17	3622 (1934.6) +	4447 (2668.3) +	3306 (1059) +	2725 (1067.2) +
PND 21	2444 (703.2) -	2797 (1150.2) -	2697 (835.9) -	2838 (1278.3) +
[b] *Overall Treatment x Session*, p=0.011				
[b] *Pairwise Treatment x Session*		NS	NS	NS
Ambulatory Activity, cumulative counts (SD) ***[+ or – indicating direction of change from value in previous session]***				
PND 13	856 (761.4)	450 (460.2)	471 (710.5)	478 (604.8)
PND 17	1549 (1036.6) +	2018 (1465.5) +	1281 (489.6) +	1112 (570.8) +
PND 21	901 (278.3) -	1068 (554.8) -	960 (347) -	1114 (615.1) +
[b] *Overall Treatment x Session*, p=0.004				
[b] *Pairwise Treatment x Session*		P=0.013	NS	NS

[a] Data used in across session analyses were obtained from Table S38 on pages 362-363 in the study final report.

[b] Analyses for Overall Treatment x Session (significance level at 0.05); Analyses for Pairwise Treatment x Session (significance level at 0.02)

2) Within Session Analyses of Motor Activity (PND 13, 17, 21, and 61):

Due to the statistical procedure used in this study, when initial overall analyses determined that sex was not a significant factor, subsequent analyses were conducted on data with male and female data pooled. Consequently, the tabulated mean total and ambulatory activity data presented in the data tables did not include mean data for males and females separately for all test sessions.

a) PND 13 Motor Activity:

On PND 13, there were significant ($p<0.013$) treatment by sex interactions for total and ambulatory counts. Therefore, this data was analyzed separately by sex. There were no statistically significant treatment by intra-session time interactions, which indicated the absence of an effect on motor activity habituation for both males and females. For total and ambulatory activity counts, there were significant ($p<0.014$) main effects of treatment in males but not in females. Post-hoc comparisons for the main effect of treatment resulted in decreases that were significant ($p<0.017$) for males at the 5 and 15 mg/kg/day dose levels for both total and ambulatory activity. There were also non-statistically significant decreases in the 100 mg/kg/day group that were of a lesser magnitude than the 5 and 15 mg/kg/day groups. When compared with the WIL historical control database, the mean values for the 5, 15, and 100 mg/kg/day group males were within the data ranges. In contrast, mean values for the concurrent control group males on PND 13 were above or approached the maximum mean values in the WIL historical control data (see page 28 in this DER document). Furthermore, there was no dose-response relationship in the decreases across the 5, 15, and 100 mg/kg/day groups (i.e., a flat dose response). Therefore, the apparent decrease in male activity was due to unusually high concurrent control group activity and was not attributed to the test substance.

b) PND 17 Motor Activity:

On PND 17, there were no treatment by sex interactions for total or ambulatory activity. Therefore, subsequent analyses were conducted on the pooled sexes (males and females combined). There was a main effect of treatment for both total and ambulatory activity, but there was not an effect on habituation (treatment by time interaction). Post-hoc group comparisons with pooled sexes data resulted in no statistically significant differences between the control group and the 5, 15, and 100 mg/kg/day groups.

c) PND 21 Motor Activity:

Locomotor activity (total activity as well as ambulatory activity counts) in F_1 animals was found to be unaffected by Fo maternal test substance administration at all dose levels when evaluated on PND 21. Since there were no treatment by sex interactions, all analyses were conducted on data from the pooled sexes (males and females combined). The absence of any statistically significant treatment by time interactions indicated that there were no treatment effects on motor activity habituation.

d) PND 61 Motor Activity:

A significant ($p<0.005$) treatment by sex by time interaction was noted for total and ambulatory counts on PND 61. Therefore, total and ambulatory counts were analyzed separately by sex. For total counts, there was a main effect of treatment ($p=0.005$) for males, without an effect on habituation (the treatment by time interaction was not statistically significant). Pairwise comparisons of the treatment effect using Dunnett's test did not result in statistically significant differences. There was no main effect of treatment or statistically significant treatment by time interaction for total counts for females. In addition, there was no main effect of treatment or effect of treatment on habituation for ambulatory counts for males and females; the treatment effect and treatment by time interactions were not statistically significant.

Mean activity data for sessions (pooled sexes) are presented in the Table 10b below.

TABLE 10b. Cumulative total and ambulatory activity data (pooled sexes) for sessions PND 13, 17, 21, and 61 (analyzed within sessions). [a]

Test Day	Dose (mg/kg/day)			
	0 (N40=20)	5 (N=20)	15 (N[Litter]=20)	100 (N[Litter]=20)
Total Activity, cumulative counts (SD)				
PND 13	2222 (1347.2)	1622 (910.6)	1593 (1322.1)	1616 (1051.1)
PND 17	3622 (1934.6)	4447 (2668.3)	3306 (1059)	2725 (1067.2)
PND 21	2444 (703.2)	2797 (1150.2)	2697 (835.9)	2838 (1278.3)
PND 61 (N= 40/dose)[b]	1001 (282.5)	1061 (244.2)	989 (226.4)	911 (299.6)
Ambulatory Activity, cumulative counts (SD)				
PND 13	856 (761.4)	450 (460.2)	471 (710.5)	478 (604.8)
PND 17	1549 (1036.6)	2018 (1465.5)	1281 (489.6)	1112 (570.8)
PND 21	901 (278.3)	1068 (554.8)	960 (347)	1114 (615.1)
PND 61 (N=40/dose) [b]	277 (108.5)	292 (95.5)	261 (91.8)	260 (132.2)

[a] Data used for within session analyses were obtained from Table S38 on pages 364-375 in the study final report.

[b] Analyses of PND 61 data did not use Litter as the statistical unit; pooling data from 20 males/group and 20 females/group resulted in the N=40/dose.

* Statistically significant at $p<0.05$

** Statistically significant at $p<0.01$

c. Auditory Startle Response/Habituation:

The auditory startle reflex test provides data on sensorimotor function and on habituation which is considered to be a simple form of learning. The major variables for this test are the amplitude of the startle reflex in response to the loud tone, and the decrease in the amplitude of the reflex (habituation) expected across the 5 blocks of 10 trials. The latency or time to peak response was recorded and the mean data was presented but was not analyzed for statistical significance. It should also be noted that a calculated quantitative index of the rate of habituation was not presented in acoustic startle response dataset; the effect of treatment on habituation of auditory startle responding was determined statistically based on the analysis of treatment by trial block interactions. Note that mean session acoustic startle maximum (peak) amplitude response (Maximum response, Newtons) and mean session startle latency to peak response (msec) for each dose group were not tabulated in the final report and thus are not presented in this DER.

Initial overall analyses of the auditory startle maximum (peak) amplitude response data for 50-trial (analyzed as 5 blocks of 10 trials) sessions on PND 20 and PND 60 revealed no statistically significant treatment by sex interactions at either test day. Therefore, the peak amplitude data for males and females were pooled together for subsequent repeated measures analyses. On PND 20, there was no statistically significant main effect of treatment or treatment by trial-block interaction on peak amplitude (the latter indicating an absence of any treatment related effect on acoustic startle habituation).

On PND 60, there was a significant main effect of treatment ($p=0.001$), but no effect on habituation as reflected by the absence of a statistically significant treatment by trial-block interaction. Post-hoc comparisons between the control group and the 5, 15, and 100 mg/kg/day groups revealed a significant ($p=0.002$) overall increase in startle peak amplitude response at the 100 mg/kg/day dose level, but not at the 5 or 15 mg/kg/day dose levels. This difference was considered treatment-related.

Mean interval acoustic startle peak amplitude response (MAX, Newtons) and mean interval startle latency to peak response (TMAX, msec) for each test block of 10 trials in the PND 20 and PND 60 test sessions are presented in Table 11 below. These data were analyzed with pooled sexes and are presented accordingly.

TABLE 11. Mean (±SD) interval acoustic startle peak response amplitude (MAX, Newtons) and latency to peak response (TMAX, msec) [b] in F_1 rats (data for pooled sexes) [a]

Dose (mg/kg/day)	Parameters [mean (SD)]	Block 1 Trial 00-10	Block 2 Trial 11-20	Block 3 Trial 21-30	Block 4 Trial 31-40	Block 5 Trial 41-50
PND 20						
0	Peak Amp.(Newtons)	2.55 (0.91)	2.03 (0.76)	1.92 (0.73)	1.68 (0.59)	1.57 (0.61)
(N=20)	*Latency (msec)*	*58.4 (3.15)*	*59.0 (2.54)*	*59.4 (2.32)*	*59.7 (3.22)*	*60.1 (3.99)*
5	Peak Amp.(Newtons)	2.24 (0.75)	1.74 (0.60	1.55 (0.55)	1.41 (0.58)	1.44 (0.57)
(N=20)	*Latency (msec)*	*58.1 (3.12)*	*58.9 (2.92)*	*59.9 (2.89)*	*59.6 (3.30)*	*60.1 (3.00)*
15	Peak Amp.(Newtons)	2.23 (0.78)	1.76 (0.64)	1.56 (0.62)	1.42 (0.62)	1.53 (0.60)
(N=20)	*Latency (msec)*	*58.9 (3.46)*	*59.0 (2.81)*	*58.9 (2.61)*	*59.4 (5.39)*	*59.7 (2.74)*
100	Peak Amp.(Newtons)	1.92 (0.65)	1.55 (0.58)	1.43 (0.65)	1.32 (0.74)	1.23 (0.53)
(N=20)	*Latency (msec)*	*58.3 (2.83)*	*58.9 (3.44)*	*60.5 (3.68)*	*61.3 (3.51)*	*61.0 (3.85)*
PND 60						
0	Peak Amp.(Newtons)	1.82 (0.96)	1.12 (0.57)	0.99 (0.47)	0.92 (0.50)	0.89 (0.50)
(N=40) [c]	*Latency (msec)*	*48.5 (12.65)*	*49.5 (13.26)*	*46.8 (11.27)*	*49.1 (13.10)*	*49.8 (10.92)*
5	Peak Amp.(Newtons)	1.86 (1.19)	1.18 (0.76)	1.03 (0.60)	0.92 (0.52)	0.85 (0.49)
(N=40)	*Latency (msec)*	*44.8 (11.96)*	*45.8 (12.27)*	*46.5 (9.50)*	*46.6 (10.83)*	*49.0 (10.82)*
15	Peak Amp.(Newtons)	1.86 (1.13)	1.26 (0.78)	1.05 (0.59)	0.96 (0.60)	0.97 (0.68)
(N=40)	*Latency (msec)*	*50.7 (11.00)*	*50.1 (12.88)*	*51.0 (11.29)*	*51.9 (11.07)*	*50.5 (10.64)*
100	Peak Amp.(Newtons)**	2.37 (1.50)	1.84 (1.14)	1.53 (0.89)	1.48 (0.96)	1.44 (0.98)
(N=40)	*Latency (msec)*	*46.1 (11.07)*	*42.6 (10.50)*	*43.8 (12.23)*	*46.0 (10.32)*	*45.0 (10.91)*

a Data were obtained from Table S39 on page 24, pages 228-230; 10 trials/block.

b *Latency data were collected and tabulated but were not analyzed for statistical significance.*

c Analyses of PND 61 data did not use Litter as the statistical unit; pooling data from 20 males/group and 20 females/group resulted in the N=40/dose.

** Significant session dose effect compared to control at p=0.002.

d) Learning and Memory Testing:

The assessment of learning/memory behaviors consisted of 3 Phases conducted over 7 days using the Manual Biel Water Maze:
Phase 1 (day 1) evaluated swimming ability and motivation to escape in 4 consecutive straight channel trials. Phase 2 (days 2-6) evaluated sequential learning with 2 days in Path A of water maze (4 trials # 1-4) and then 3 days in Path B (6 trials # 5-10). Phase 3 (day 7) evaluated memory retention of Path 1 (2 trials #11-12).

Based on a parametric one-way ANOVA of the mean escape times in the straight channel of the Biel water maze for both the PND 22 and PND 62 test sessions, there were no significant treatment related intergroup differences in the F_1 offspring tested at PND 22 (Subset B offspring) or PND 62 (Subset A offspring). This indicated that the swimming ability and level of motivation to escape from the water maze of PND 22 and PND 62 male and female F_1 offspring were unaffected by F_o maternal dosing with 5, 15, and 100

mg/kg/day test agent.

On PND 22, there were no statistically significant main effects of treatment and no treatment by trial or sex interactions on Path A learning or memory for both escape time (time to locate platform) and errors. There was a similar absence of statistical significance for a main effect of treatment or treatment by trial or sex interactions and for Path B escape time. For Path B number of errors, there was a significant (p=0.041) treatment by sex by trial interaction. Therefore, the numbers of errors were analyzed separately by sex. In males, there was no statistically significant treatment by trial interaction or treatment effect. For females, however, there was a significant (p=0.003) treatment by trial interaction. The post-hoc pairwise interaction comparisons resulted in significant differences for the 5 (p=0.02) and 100 (p=0.004) mg/kg/day group females, when compared with the control group; the errors for the 15 mg/kg/day group male and female F_1 offspring were similar to controls. In general, the significant differences at the 5 and 100 mg/kg/day dose levels involved fewer errors committed by the F_1 females (i.e., the animals learned faster) throughout most of the trials on Path B, compared to the control group. Most of the mean errors (Path B) for the treated groups were within 1 standard deviation of the historical control data for PND 22 females (see historical control values on page # in this DER document). Contrarily, most of the mean errors (Path B) for the control group were more than 1 standard deviation higher than the historical control data for PND 22 females. In the final report the investigator considered these differences in mean numbers of errors between the control and the 5 and 100 mg/kg/day group females not to be treatment related in view of the absence of a dose-response and given that the apparent differences in F_1 female Path B errors, but not males, occurred during PND 22, a period when sex-specific changes are not expected due to the immature state of the animals. Another compelling rationale for not considering these differences to be treatment related, which was not mentioned in the final report, is based on the fact that not only were most of the female mean errors (Path B) for the treated groups within 1 standard deviation of the historical control data for PND 22 females but also most of the female errors (Path B) in the 0 mg/kg/day control group were more than one standard deviation higher than the historical control data for PND 22 females (see historical control values on pages 28-29 in this DER document). Consequently, in the opinion of this reviewer, the differences between the errors (Path B) in the 5 and 100 mg/kg/day females and the unusually higher error scores in the control should be considered false positives and not treatment related effects.

A discrepancy in the final report that was noted in the presentation of the Biel water maze data for PND 22 in Table S40 was that all mean values of escape time and errors for the 5 mg/kg/day dose group, when pooled sex data were displayed, were reported as having an N=21. All other PND 22 dose groups and the control group displayed the pooled sex data with the appropriate N=20. No explanation for this discrepancy is presented in the final report and it may possibly have been a typographical error.

On PND 62, there were no statistically significant effects on the mean escape times during the learning (Path A and B) and memory trials for the F_1 males and females in the 5, 15, and 100 mg/kg/day groups, when compared with the control group. The mean numbers of errors committed during learning and memory trials were also similar across all groups.

The swimming ability and motivation data and the learning/memory data for each testing age are presented in Table12a (PND22) and Table12b (PND62), below.

TABLE 12a. Biel swimming trials [mean data (S.D.)] for PND 22 F_1 offspring [ab]

Phase / Parameter	Trial	Parameter [mean (SD)]	Dose (mg/kg/day) 0 N=20	5 N=21 [c]	15 N=20	100 N=20
PND 22 [Subset B offspring] (pooled sexes unless indicated otherwise) [b]						
Phase 1 / Swim Ability & Motivation	(Day 1)	Straight Channel Escape Time [sec (SD)]	9.1 (3.05)	8.1 (2.02)	8.13 (2.02)	9.59 (2.72)
Phase 2/ Learning Path A	1	Escape Time [sec (SD)]	74.92 (27.82)	82.75 (39.58)	78.02 (37.46)	74.51 (36.11)
		Errors [number (SD)]	16 (7.0)	18 (10.3)	17 (8.4)	15 (7.9)
	2	Escape Time [sec (SD)]	57.60 (30.85)	65.98 (24.81)	63.40 (34.52)	66.58 (31.53)
		Errors [number (SD)]	12 (7.2)	14 (7.0)	13 (8.4)	15 (8.8)
	3	Escape Time [sec (SD)]	40.64 (33.66)	52.15 (29.22)	49.52 (29.84)	60.84 (31.37)
		Errors [number (SD)]	8 (7.3)	11 (7.3)	11 (8.1)	15 (10.0)
	4	Escape Time [sec (SD)]	41.18 (28.56)	45.30 (26.15)	34.46 (20.57)	53.44 (33.48)
		Errors [number (SD)]	9 (7.5)	9 (6.7)	6 (4.8)	12 (9.1)
Phase 2/ Learning Path B **	5	Escape Time [sec (SD)]	149.69 (28.99)	150.35 (36.05)	146.33 (32.44)	139.53 (50.41)
		Errors [number (SD)] N=20/sex/group	M: 37 (14.5) F: 38 (16.0)	M: 37 (10.7) **F: 34 (17.3)**	M: 33 (15.1) F: 36 (13.8)	M: 35 (14.9) **F: 31 (14.9)**
	6	Escape Time [sec (SD)]	134.85 (35.84)	115.21 (46.46)	128.22 (38.49)	132.51 (37.51)
		Errors [number (SD)] N=20/sex/group	M: 26 (13.1) F: 33 (10.6)	M: 27 (16.3) **F: 21 (13.1)**	M: 28 (12.4) F: 28 (13.1)	M: 27 (14.1) **F: 33 (12.7)**
	7	Escape Time [sec (SD)]	109.89 (44.28)	110.51 (50.23)	90.26 (41.60)	107.43 (37.96)
		Errors [number (SD)] N=20/sex/group	M: 21 (16.4) F: 30 (19.7)	M: 26 (16.3) **F: 21 (15.5)**	M: 18 (12.2) F: 21 (15.9)	M: 27 (16.9) **F: 22 (13.9)**
	8	Escape Time [sec (SD)]	103.14 (44.01)	100.72 (46.65)	78.12 (33.17)	66.56 (34.55)
		Errors [number (SD)] N=20/sex/group	M: 19 (13.2) F: 26 (15.4)	M: 18 (15.0) **F: 22 (15.4)**	M: 16 (12.4) F: 16 (12.8)	M: 13 (13.3) **F: 12 (12.7)**
	9	Escape Time [sec (SD)]	86.22 (51.20)	83.81 (51.67)	63.34 (32.66)	71.58 (45.85)
		Errors [number (SD)] N=20/sex/group	M: 15 (15.3) F: 24 (17.7)	M: 21 (15.9) **F: 13 (13.5)**	M: 12 (12.3) F: 15 (14.1)	M: 14 (13.8) **F: 17 (17.0)**
	10	Escape Time [sec (SD)]	49.38 (43.71)	54.21 (38.20)	61.30 (35.83)	58.19 (29.36)
		Errors [number (SD)] N=20/sex/group	M: 11 (14.5) F: 8 (9.6)	M: 7 (10.2) **F: 13 (14.2)**	M: 16 (16.1) F: 10 (11.4)	M: 9 (9.9) **F: 16 (11.6)**
Phase 3 / Memory-Retention Path A	11	Escape Time [sec (SD)]	75.10 (33.41)	86.76 (38.33)	77.82 (27.57)	66.22 (24.29)
		Errors [number (SD)]	22 (11.4)	26 (13.5)	22 (8.8)	20 (8.7)
	12	Escape Time [sec (SD)]	48.73 (16.51)	52.43 (29.68)	53.41 (26.60)	48.52 (27.17)
		Errors [number (SD)]	12 (4.5)	12 (8.4)	13 (8.2)	12 (8.5)

a Data obtained from Tables S40 on pages 380-392 in the study final report.

b Since initial overall analyses for all Trial Groups and Parameters, except for Errors Learning Path B/Trials 5 to 10, revealed that sex was not a significant interaction factor, those data were subsequently analyzed with sexes pooled with their mean data entries representing N=20 animals/dose group for the PND 22 session with litter as the statistical unit (for these datasets separate male/female mean data were not provided). However, since sex was a significant factor for Errors Learning Path B/Trials 5 to 10, male and female data for those trials were analyzed separately and mean data for males and females (both at N=20 animals/dose group) were provided and included in this Table.

c In the PND 22 session, all mean values for the 5 mg/kg/day dose group using pooled sex data were displayed in the final report Table S40 with an N value of 21. All other dose groups and controls using pooled sex data in the PND 22 session were displayed in the final report with the appropriate N = 20. No explanation for this discrepancy is presented in the final report and may possibly represent a typographical error.

Path A = forward through maze; Path B = reverse through maze; Time = mean time to escape; Error = all four feet into an incorrect channel.

** Significant Treatment x Trial effect for females only in both the 5 mg/kg/day (p=0.02) and 100 mg/kg/day (p=0.004) groups; but both were considered to be false positives due to unusually high Error scores for the concomitant female controls and consequently not treatment related effects

TABLE 12b. Biel swimming trials [mean data (S.D.)] for PND 62 F_1 offspring [ab]

Phase / Parameter	Trial	Parameter [mean (SD)]	Dose (mg/kg/day) 0 N=40 [b]	5 N=40	15 N=40	100 N=40
PND 62 (Subset A offspring) (pooled sexes)						
Phase 1 / Swim Ability & Motivation	(day 1)	Straight Channel Escape Time, mean sec (SD)	5.28 (1.58)	5.79 (2.04)	6.04 (3.01)	5.93 (2.73)
Phase 2/ Learning Path A	1	Escape Time [sec (SD)]	54.70 (35.85)	74.27 (52.67)	67.88 (24.10)	78.69 (55.51)
		Errors [number (SD)]	12 (8.2)	16 (11.2)	15 (11.4)	17 (12.2)
	2	Escape Time [sec (SD)]	58.98 (43.43)	67.69 (48.12)	68.03 (49.78)	69.38 (48.35)
		Errors [number (SD)]	14 (10.1)	15 (11.1)	16 (12.9)	17 (12.3)
	3	Escape Time [sec (SD)]	45.87 (36.79)	51.30 (43.63)	47.37 (40.70)	48.08 (43.97)
		Errors [number (SD)]	13 (10.7)	13 (12)	11 (10.7)	13 (15.1)
	4	Escape Time [sec (SD)]	24.59 (24.75)	30.36 (32.66)	27.75 (24.33)	32.30 (32.89)
		Errors [number (SD)]	5 (7.5)	6 (8.5)	6 (6)	8 (10.2)
Phase 2/ Learning Path B	5	Escape Time [sec (SD)]	123.62 (58.20)	126.91 (56.70)	127.27 (59.16)	139.65 (54.51)
		Errors [number (SD)]	27 (13.6)	25 (12.1)	26 (12.9)	28 (13.10
	6	Escape Time [sec (SD)]	78.22 (61.15)	88.92 (58.39)	104.98 (66.35)	87.89 (48.93)
		Errors [number (SD)]	17 (13.50	17 (12.2)	21 (14.6)	18 (11.4)
	7	Escape Time [sec (SD)]	70.90 (49.74)	61.02 (53.95)	67.19 (53.08)	69 (47.31)
		Errors [number (SD)]	16 (11.5)	12 (13.2)	13 (10.6)	15 (11.9)
	8	Escape Time [sec (SD)]	41.68 (45.56)	39.98 (39.44)	43.28 (47.15)	42.20 (40.86)
		Errors [number (SD)]	8 (9.9)	8 (10.4)	7 (8.5)	9 (11.2)
	9	Escape Time [sec (SD)]	28.23 (23.54)	28.67 (22.28)	41.38 (39.89)	30.19 (32.89)
		Errors [number (SD)]	6 (8.1)	5 (5.5)	7 (8.5)	6 (10.3)
	10	Escape Time [sec (SD)]	22.87 (19.53)	24.94 (24.68)	23.92 (17.18)	20.91 (21.35)
		Errors [number (SD)]	5 (7.5)	4 (5.4)	5 (8.7)	4 (9.3)
Phase 3 / Memory-Retention Path A	11	Escape Time [sec (SD)]	71.11 (55.55)	76.71 (51.90)	85.30 (52.65)	59.36 (41.85)
		Errors [number (SD)]	19 (15)	19 (13.8)	21 (14.1)	15 (11.3)
	12	Escape Time [sec (SD)]	42.15 (31.90)	52.74 (46.23)	34.64 (26.95)	46.43 (42.01)
		Errors [number (SD)]	10 (7)	11 (10.9)	7 (6.4)	11 (11.7)

a Data obtained from Tables S 41 on pages 393-401 in the study final report.

b Since initial overall analyses for all PND 62 Trial Groups and Parameters revealed that sex was not a significant interaction factor, those data were subsequently analyzed with sexes pooled. Since PND 62 session data analyses did not use litter as the statistical unit the mean values for these data typically represent N=40 animals/dose group; separate male/female mean data were not provided. There were several exceptions to the N=40/dose group; an N=39 was used for the Trial 4/EscapeTime in the 15 mg/kg/day group, Trial 10/Escape Time in 15 mg/kg/day group, and Trial 12/Errors in the 5 mg/kg/day group.

Path A = forward through maze; Path B = reverse through maze; Time = mean time to escape; Error = all four feet into an incorrect channel.

6. Postmortem Results: General Macroscopic Examinations:

Necropsies of Pups Found Dead or Euthanized *in Extremis*

No internal findings that could be attributed to maternal administration of the test substance at any dose level were noted at the necropsies of pups that were found dead. One female in the 5 mg/kg/day group was euthanized in extremis due to a mechanical injury; no test substance-related internal findings were observed for this female.

Necropsies of Pups on PND 21 Not Selected For Neuropathological Evaluation or Behavioral Testing

No internal findings that could be attributed to Fo maternal test substance administration were noted at the necropsy of pups euthanized on PND 21.

a. Animals euthanized following attainment of sexual developmental landmarks (Subset B):
There were no internal findings noted for F_1 male and female offspring euthanized following attainment of sexual developmental landmarks (N=20/sex/group).

b. Animals not selected for neuropathology/brain weight/brain measurements and euthanized at study termination (Subset A):
At the PND 72 necropsies of F_1 male and female animals not selected for neuropathology and brain weights (N=5/sex/group), no internal gross findings related to F_0 maternal test substance administration were observed.

7. Neuropathology and Related Assessments (PND 21 – Subset C; PND 72 – Subset A):

a. Macroscopic Examinations:

There were no test substance-related macroscopic changes observed in the brain or spinal animals (Subset C) selected for evaluation on PND 21 (N=15/sex/group). Various findings were noted in the brain at 100 mg/kg/day, including dark red areas/discoloration and depressed areas. These macroscopic findings were observed in single animals, had no correlating microscopic findings, and/or were observed similarly in the control group. Therefore, these findings were considered spontaneous, incidental occurrences.

There were no macroscopic changes observed in the brain of the F_1 animals (Subset A) selected for evaluation on PND 72 (N=15/sex/group).

b. Brain Weights/Brain Measurements:

A significantly ($p<0.01$) lower mean brain weight (5% decrease compared with control) was noted in the 100 mg/kg/day group males at PND 21 (Subset C). This group also had a significantly ($p<0.01$) lower mean final body weight (20% decrease compared with control) which resulted in a relative brain weight (grams brain/100 grams body weight) that was significantly ($p<0.01$) higher than the control group. The PND 21 females in the 100 mg/kg/day dose group did not have significantly lower brain weights but they were found to have significantly ($p<0.05$) lower final body weights (12% lower than control) which resulted in a significantly ($p<0.05$) higher relative brain weight, when compared with controls. These results indicated that the toxicity at the 100 mg/kg/day dose level was generalized rather than specifically targeted to the nervous system; the lower mean brain weights in males were associated with the lower body weights and the higher brain to body weight ratios in both male and female offspring reflected the lowered body weights together with the relatively smaller decrease in brain weight or normal brain weight.

On PND 72 (Subset A), the F_1 males in the 100 mg/kg/day dose group had brain weights similar to controls but they were found to have significantly ($p<0.01$) lower final body weights (10% lower than control) with resulting significantly ($p<0.01$) higher relative brain weights, when compared with controls. Note that these PND 72

results were not identified or discussed in the final report. In the opinion of the reviewer these results indicated that the toxicity at the 100 mg/kg/day dose level was generalized rather than specifically targeted to the nervous system; the higher relative brain weights reflected the decreased final body weights together with the normal brain weight. There were no brain weight or relative brain weight changes on PND 72 in the 100 mg/kg/day females or in either sex at at the 5 and 15 mg/kg/day dose levels. There were also no effects of treatment at any dose level on brain measurements of brain length and brain width in the PND 72 male and female offspring.

Overall, the test substance at all dose levels (5, 15 and 100 mg/kg/day) was considered to have induced no direct treatment related effects on brain weights and brain measurements for PND 21 and PND 72 F_1 male and female offspring. However, the 100 mg/kg/day dose level did elicit a generalized toxicity in the PND 21 male and female and PND 72 male offspring that included significant decreased final body weights (PND 21 males and females, and PND 72 males) and a corresponding but smaller decrease in brain weight (PND 21 males only), but no direct neurotoxic effect.

The mean brain weight and brain measurement data for the PND 21 and PND 72 F_1 offspring are presented in Table 13 below.

TABLE 13. Mean (±SD) brain weight and brain measurement data for F_1 offspring at PND 21 and PND 72 [a]

Parameter	Dose (mg/kg/day)			
	0 N=15	5 N=15	15 N=15	100 N=15
PND 21 (Subset C F_1 Offspring)				
MALES				
Final body weight (g)	55 (6.7)	50 (6.1)*	54 (5.2)	44 (4.6)**
Brain weight (g)	1.6798 (0.064)	1.6298 (0.080)	1.6658 (0.047)	1.5852 (0.066)+
Brain (g) /body weight (100 g) ratio	3.100 (0.282)	3.325 (0.322)	3.109 (0.284)	3.619 (0.356)**
Brain Length (mm)	18.0 (0.46)	18.1 (0.43)	18.2 (0.25)	18.0 (0.54)
Brain Width (mm)	14.7 (0.45)	14.6 (0.47)	14.7 (0.33)	14.4 (0.28)
FEMALES				
Terminal body weight (g)	50 (6.0)	48 (5.3)	52 (5.9)	44 (5.0)*
Brain weight (g)	1.5682 ((0.142)	1.5617 (0.077)	1.6092 (0.074)	1.5287 (0.033)
Brain (g) /body weight (100 g) ratio	3.168 (0.370)	3.308 (0.284)	3.158 (0.396)	3.529 (0.372)*
Brain Length (mm)	18.0 (0.74)	18.0 (0.42)	18.1 (0.59)	17.9 (0.45)
Brain Width (mm)	14.3 (0.44)	14.5 (0.43)	14.3 (0.34)	14.1 (0.32)
PND 72 (Subset A F_1 Offspring)				
MALES				
Final body weight (g)	444 (33.1)	428 (40.6)	445 (39.1)	399 (37.7)**
Brain weight (g)	2.2653 (0.111)	2.2727 (0.128)	2.3047 (0.073)	2.2047 (0.087)
Brain (g) /body weight (100 g) ratio	0.512 (0.0272)	0.534 (0.0313)	0.521 (0.0414)	0.556 (0.0422)**
Brain Length (mm)	21.4 (0.38)	21.5 (0.39)	21.5 (0.39)	21.3 (0.41)
Brain Width (mm)	15.5 (0.31)	15.4 (0.42)	15.5 (0.23)	15.3 (0.31)
FEMALES				
Terminal body weight (g)	258 (21.8)	274 (22.6)	272 (22.6)	252 (28.4)
Brain weight (g)	2.0740 (0.094)	2.0920 (0.109)	2.1293 (0.077)	2.0133 (0.136)
Brain (g) /body weight (100 g) ratio	0.807 (0.0591)	0.767 (0.0590)	0.787 (0.0563)	0.806 (0.0740)
Brain Length (mm)	20.8 (0.37)	20.9 (0.39)	21 (0.32)	20.7 (0.30)
Brain Width (mm)	15 (0.33)	14.9 (0.23)	15 (0.31)	14.8 (0.34)

[a] Data obtained from Tables S47 and S48 on pages 408-411 and Tables S52 and S53on pages 426-429 in the study final report.

+ Statistically different from control, p<0.001 with significance level of [p= 0.05/sqrt (number of comparisons)]

* Statistically different from control, p<0.05

** Statistically different from control, p<0.01

c. Qualitative Histopathology and Brain Morphometry:

1) Histopathology:

Note that the procedures described in the Materials and Methods section of the final report and in the Pathology Report (Appendix F of the final report) a maximum of N=10 pup/sex/group were to be used for the histopathology examinations at each age of examination PND 21 and PND 72. As reported in the final report, at the PND 21 (Subset C) examination the 0, 5 and 15 mg/kg/day dose groups each had N=10/sex/group, but the 100 mg/kg/day group had N=11 males and N=13 females. At the PND 72 (Subset A) examination the 5, 15 and 100 mg/kg/day groups each had N=10/sex/group and the 0

mg/kg/day female group had the appropriate N=10 animals, but the 0 mg/kg/day group of males consisted of N=11 animals. No explanation for these discrepancies was provided.

There were no treatment related microscopic findings in the brains of the PND 21 animals (Subset C). Two PND 21 pups (one control female and one 100 mg/kg/day male) had developmental anomalies unilaterally in the brains which involved partial agenesis of the hippocampus and dysplasia and attenuation of the overlying cortex. These findings were considered spontaneous because of their occurrence in a control group female. Other microscopic observations in the brains of PND 21 rats included unilateral embryonal cell nests in 2 males and 1 female from the 5 mg/kg/day group, 1 male and 2 females from the 15 mg/kg/day group, and 1 male and 1 female from the 100 mg/kg/day group. These nests of embryonal cells most closely resembled the cells of the normal subependymal zone and typically occurred in the lateral caudoputamen close to the location of the lateral ventricles. Although there were no control group animals with this finding in the present study, the WIL Research historical control database (*reviewer could find no final report Appendix that contained theWIL historical histopathology control data*) includes 'ectopic tissue' in the basal ganglia, an alteration that is identical to that of 'embryonal cell nests.' Ectopic tissue (identical to embryonal cell nests) was reported to be present in 1/10 males and 1/10 females in 1 study. In the present study, embryonal cell nests had no dose relationship and were not considered treatment related. Edema, with separation of the tissue in white matter tracts, occurred unilaterally in one 5 mg/kg/day female and one 100 mg/kg/day female. There was no dose relationship and this finding was not considered related to administration of the test substance. Two 100 mg/kg/day group males had minimal dilatation of the lateral ventricle(s) which was not considered treatment related because of the common occurrence of such alterations.

There were no treatment related microscopic findings in the brains of the PND 72 (Subset A) animals. Microscopic observations in the brains of PND 72 rats included minimal dilatation of the lateral ventricle, typically unilateral. Incidences for the control, 5, 15, and 100 mg/kg/day groups respectively were 0, 1, 2, and 1 for males and 0, 1, 0, and 1 for females. The lack of a dose-response was consistent with dilatation being either spontaneous or related to incidental over-perfusion. Similarly, remaining histologic changes in the examined tissues were considered to be incidental findings or related to some aspect of experimental manipulation other than administration of the test substance. There was no test substance-related alteration in the prevalence, severity, or histologic character of those incidental tissue alterations. Minimal axonal degeneration in the peripheral nerves had similar incidences between control and 100 mg/kg/day group animals.

2) <u>Brain Morphometry:</u>

There was no evidence of disturbed tissue architecture, disordered migration or development, or altered cell populations in the brains of the PND 21 male and female animals (Subset C) at any treatment level. The mean measurement for the base of cerebellar lobule 9 in 100 mg/kg/day group PND 21 females was approximately 10% greater than the mean for control females but this difference was only 63 microns. Further, this measurement was not different in PND 21 males. An increased length is unlikely to reflect a deficiency of myelination or cell migration. Together with the variance in the control group (0.009), the absence of findings in PND 21 males, the lack of a dose-response relationship, lack of a similar change in the height of the cerebellum, and absence of histopathology findings indicates that these perturbations from control are not biologically meaningful (See Table 14). Similar considerations apply to other measurements that had differences of 5% or greater from control. None were considered related to administration of the test substance. Rather, measurements were similar between all 4 groups.

There were no treatment related effects on brain morphometry, such as disturbed tissue architecture, disordered migration or development, or altered cell populations, in the brains of the PND 72 male and female animals (Subset A) at any dose level.

The morphometric findings for F1 male and female animals at PND 21 and PND 72 are presented in the following Table 14.

TABLE 14. Mean (µm ± SD) morphometric data [a]

Parameter	Dose (mg/kg/day)			
	0 mg/kg/day	5 mg/kg/day	15 mg/kg/day	100 mg/kg/day
	MALES [c]			
	PND 21 (Subset C Males) Mean µm (± SD)			
LEVEL 1:				
- [b] V HT Hemisphere	7163 (280.6)	6926 (186.5) N=8	7183 (257.0)	7156 (395.6) N=8
- V HT (thickness) Cortex	1767 (96.2)	1742 (93.7)	1785 (62.5)	1784 (47.0)
LEVEL 3:				
- Radial Thickness Cortex	1705 (109.7)	1760 (153.6)	1729 (108.1)	1783 (139.5)
- V HT btw Hippocampal Pyramidal Neuron Layers	887 (54.3)	932 (78.5)	923 (69.3)	869 (81.7)
- V HT Dentate Hilus	494 (31.3)	519 (52.7)	528 (39.2)	497 (39.2) N=9
LEVEL 5:				
- HT Cerebellum	4874 (284.5) N=9	4942 (254.2)	4963 (129.0)	4868 (172.4)
- Thickness Base of Cerebellar Lobule 9	644 (78.1)	650 (82.9)	641 (55.1)	627 (73.8)
	PND 72 (Subset A Males) Mean µm (± SD)			
LEVEL 2:				
- HT Hemisphere	8395 (239.4)	8219 (231.5)	8359 (328.7)	8239 (373.5)
- V HT (thickness) Cortex	1680 (80.8)	1660 (61.9)	1697 (83.3)	1696 (106.8)
LEVEL 3:				
- Radial Thickness Cortex	1774 (99.4)	1760 (115.0)	1730 (85.2)	1675 (99.8) N=9
- V HT btw Hippocampal Pyramidal Neuron Layers	997 (72.1)	1033 (67.0)	1010 (56.0)	1028 (68.8)
- V HT Dentate Hilus	544 (46.7)	555 (43.2)	533 (37.1)	553 (47.7) N=9
LEVEL 5:				
- HT Cerebellum	5249 (177.5)	5266 (205.1)	5234 (266.5)	5173 (253.9) N=9
- Thickness Base of Cerebellar Lobule 9	756 (49.3)	747 (63.4)	734 (36.6)	743 (30.6) N=8

(Table 17 continued below)

(continued: TABLE 17. Mean (µm ± SD) morphometric data [a])

Parameter	Dose (mg/kg/day)			
	0 mg/kg/day	5 mg/kg/day	15 mg/kg/day	100 mg/kg/day
	FEMALES [c]			

Parameter	Dose (mg/kg/day)			
	0 mg/kg/day	5 mg/kg/day	15 mg/kg/day	100 mg/kg/day
	PND 21 (Subset C Females) Mean µm (± SD)			
LEVEL 1:				
- [b]V HT Hemisphere	6946 (457.8)	7009 (239.5)	7116 (162.8)	7077 (285.4)
- V HT (thickness) Cortex	1730 (94.8)	1785 (75.5)	1798 (60.7)	1776 (89.1)
LEVEL 3:				
- Radial Thickness Cortex	1686 (121.0) N=9	1692 (113.7)	1698 (57.1)	1719 (84.0)
- V HT btw Hippocampal Pyramidal Neuron Layers	916 (57.3) N=9	940 (92.7)	947 (64.2)	976 (45.7)
- V HT Dentate Hilus	514 (29.9) N=9	531 (52.5)	538 (37.2)	542 (50.2)
LEVEL 5:				
- HT Cerebellum	4557 (334.3)	4635 (208.2)	4591 (99.2)	4519 (107.1)
- Thickness Base of Cerebellar Lobule 9	610 (89.0) N=9	671 (53.5) N=9	651 (35.2) N=9	673 (49.0)
	PND 72 (Subset A Females) Mean µm (± SD)			
LEVEL 2:				
- HT Hemisphere	8093 (237.6)	8117 (300.6)	8162 (277.4)	7914 (201.4)
- V HT (thickness) Cortex	1685 (55.3)	1727 (96.4)	1706 (83.1)	1681 (77.6)
LEVEL 3:				
- Radial Thickness Cortex	1747 (59.0)	1814 (103.0)	1749 (66.0)	1719 (90.4)
- V HT btw Hippocampal Pyramidal Neuron Layers	940 (57.6)	951 (88.4)	959 (65.5)	952 (39.4)
- V HT Dentate Hilus	508 (27.5)	523 (52.5)	520 (28.0)	513 (31.3)
LEVEL 5:				
- HT Cerebellum	5076 (157.7) N=8	5103 (265.0) N=8	5118 (125.7) N=8	5018 (275.0) N=8
- Thickness Base of Cerebellar Lobule 9	720 (69.5) N=8	753 (55.0) N=8	726 (71.5) N=8	746 (58.3) N=8

[a] Data obtained from Table S50 on pages 418-423 (PND 21 data) and Table S55 on pages 444-449 (PND 72 data) in the study final report.

[b] V = vertical; HT = height

[c] N= 10 /sex/dose unless indicated otherwise

* Statistical significance based on: Pairwise p-value under individual dose group tested at [0.05/sqrt (number of comparisons)] significance level.

III. DISCUSSION and CONCLUSIONS:

A. INVESTIGATORS' CONCLUSIONS:

In the F_1 generation, decrements in mean body weights and body weight gains, increased maximum response to the auditory startle stimulus, decreased total and ambulatory motor activity counts, and decreased grip strength were observed at 100 mg/kg/day. These effects were considered consistent with non-specific developmental delay related to maternal toxicity and poor maternal care. Based on these results, the no-observed adverse- effect level (NOAEL) for F_1 developmental neurotoxicity was considered to be 15 mg/kg/day.

In the F_0 maternal animals, adverse clinical findings were observed at 15 and 100 mg/kg/day

during the post-dosing observations; similar findings were observed during the detailed clinical observations. These findings included sitting with the head held low, hypoactivity, rocking, lurching, or swaying while ambulating, flattened body, piloerection, tremors/convulsions, slightly drooping eyelids, decreased respiration, dilated pupils, lacrimation, and/or yellow/clear material on various body surfaces. Observations indicative of a deficit in maternal care were also noted during the post-dosing evaluations at 100 mg/kg/day and included increased incidences of the dam away from the nest (but not eating, drinking, grooming, or tending to the litter) and an increased number of pups outside of the nest. In addition, decrements in body weight and body weight gains, with corresponding reductions in food consumption, were noted at 100 mg/kg/day during gestation and lactation. Based on this, the NOAEL for F_0 systemic toxicity of demiditraz when administered orally was considered to be 5 mg/kg/day. Based on decreased postnatal survival, pup body weight, and pup body weight gains at 100 mg/kg/day, the NOAEL for F_1 neonatal toxicity was considered to be 15 mg/kg/day.

B. <u>REVIEWER COMMENTS</u>:

Bred female Crl:CD(SD) rats (N=25/group) were administered the test substance, demiditraz (PF-03814927), at dosage levels of 0 (vehicle), 5, 15, or 100 mg/kg/day orally by gavage from gestation day 6 through lactaton day 20.

Post-dose treatment related clinical effects were observed in all F_o females in the 100 mg/kg/day group generally throughout the GD 6 – LD 20 treatment period, occurring at 15-30 minutes after dosing and continuing with decreases incidences into the 2-hour observation period. These effects included behavior/CNS-related findings (sitting with the head held low, hypoactivity, rocking, lurching, or swaying while ambulating, flattened body, piloerection, tremors/convulsions, and slightly drooping eyelids), cardio-pulmonary findings (decreased respiration), and material/autonomic-related findings (yellow material on various body surfaces, clear material around the mouth/salivation, lacrimation, and dilated pupils). These findings were not observed prior to the daily dosing. At 15 mg/kg/day, increased incidences of sitting with the head held low, hypoactivity, slightly drooping eyelids, dilated pupils, a flattened body, decreased respiration, and clear material around the mouth were observed at 15-30 minutes post-dosing, but generally did not persist into the 2-hour observation period. No relevant clinical findings were observed for the 5 mg/kg/day dose group.

Maternal behaviors of the F_0 females in the 100 mg/kg/day dose group also appeared to be affected based on a decreased incidence of females on the nest, a higher incidence of females away from the nest, and higher incidences of 1-3 pups and more than 3 pups outside the nest, all of which were noted at 15-30 minutes post-dosing and continuing to a lesser extent into the 2-hour post-dosing observations. These differences in maternal care between control and the 100 mg/kg/day groups were not as notable in the daily observations prior to dosing. The investigators reported that "The maternal care behavior for the 5 and 15 mg/kg/day groups was similar to the control group at approximately 15-30 minutes post-dosing, 2 hours post-dosing, and at the daily examinations." However, it would be more accurate to note that certain measures of maternal care did appear to be slightly affected in the 5 and 15 mg/kg/day groups of F_0 females. Relative to concurrent controls, there were slightly increased incidences of F_0 females being away from the nest and of scattered litters with 1-3 pups outside the nest at 15-30 minutes post-dosing for both the 5 and 15 mg/kg/day dose groups. These effects appeared to continue to some extent into the 2-hour post dose period mainly for the 15 mg/kg/day group. The suggested decrement in maternal care based on the

observations noted at the 100 mg/kg/day dose level and the tendency for a slight effect on maternal care at the 15 mg/kg/day dose level may be consistent with the extent to which clinical observations were affected in the F_0 females at each of these dose levels. However, in the absence of any notable clinical observations or other treatment related effects in the 5 mg/kg/day group of F_0 females, the apparent slight changes in maternal behavior at this dose level are not considered biologically relevant.

In the detailed clinical observations assessed on GD 10 and 15 and LD 10 and 20 prior to the daily dosing, significantly more Fo females in the 100 mg/kg/day group than in the control group were observed in the home cage with a flattened posture or sitting with the head held low, low or very low arousal, and correspondingly fewer females sitting or standing normally and having normal arousal on all test days; occasional other findings included tremor, decreased rearing, and decreased alertness. The females in the 15 mg/kg/day group exhibited no significant changes in home cage parameters on GD 10 but by GD 15 there were significantly more females in this group than controls that were sitting with their heads held low and fewer females that were alert or sitting or standing normally. On LD 10 and 20, there were still several female sitting with their heads held low and with decreased alertness but these differences were no longer significantly different from control. On LD 20 , females in the 15 mg/kg/day group exhibited a small but significant decrease in incidence of rearing (3 in dosed group vs 11 in control). This reviewer did not agree with the investigators' conclusion that, since most females in that dose group were sitting/standing normally, this decrease in rearing was not treatment related. Since this decrease in rearing in the 15 mg/kg/day group compared with control, although small, was significant and was consistent with the even greater decrease in home cage rearing at the 100 mg/kg/day dose level, there viewer concluded that this effect at the 15 mg/kg/day dose level is treatment related. No treatment related findings were observed in home cage parameters on any day of testing (i.e., GD 10 and 15 and LD 10 and 20) for the 5 mg/kg/day dose group.

The detailed clinical observations made during handling revealed significantly more females in the 100 mg/kg/day group than in the control group with slight lacrimation, slight salivation, slight piloerection, slightly drooping eyelids, and decreased respiration on various test days during gestation and lactation. No treatment related findings were observed in the handling observations for either the 5 mg/kg/day or 15 mg/kg/day dose groups.

In the open field portion of the detailed clinical observations, significantly more females in the 100 mg/kg/day group, compared with the control group, exhibited ataxia, slightly impaired mobility, and increased urinations on each test day during gestation and lactation. A limited non-significant number of females in the 100 mg/kg/day dose group also exhibited moderately impaired mobility, tremors, dragging body, slight to moderately coarse tremors, and a slight increase in backing behavior at various test days. The reviewer does not agree with the investigator's conclusion that the slight increase in backing behavior was not treatment related, since this statistically significant, although small change in open field 'backing counts' is consistent with the various other treatment related clinical observations at this dose level. At 15 mg/kg/day, no test substance-related open field observations were noted on GD 10. On each of the remaining test days, single occurrences of slightly impaired mobility and ataxia were observed for the 15 mg/kg/day group which, although not statistically significant, were viewed as related to treatment given the relationship to other findings observed at 100 mg/kg/day. Slightly higher urination counts was also observed for the 15 mg/kg/day group compared with controls on LD 20. No treatment related findings

were observed in the 5 mg/kg/day group for any open field parameter on any test day. Also, no treatment related effects on pupillary response were noted for F_0 females on any test day during gestation or lactation at any dose level. In general, the detailed clinical observations described above basically correlated with observations noted during the daily post-dosing evaluations.

The F_0 female body weights and body weight gains in the 100 mg/kg/day dose group were significantly but only slightly smaller than controls, during gestation. Mean body weights and body weight gains during gestation were not affected at either the 5 or 15 mg/kg/day dose levels. In one instance mean body weight gain for the 5 mg/kg/day dose group was statistically lower than control ($p<0.01$) but, since this decrease was transient and not observed in a dose-dependent manner, it was considered not to be treatment related. Consistent with the lower body weight gains and body weights of the F_0 females in the 100 mg/kg/day group, significantly reduced mean food consumption relative to control was also noted for the 100 mg/kg/day dose group during gestation. Mean food consumption during gestation was unaffected by either the 5 and 15 mg/kg/day dose treatments.

Although F_0 female mean body weight gains for the 100 mg/kg/day group were occasionally somewhat lower than control during the first two weeks of lactation, mean body weight gains at this dose level were similar to the control group during the remainder of the lactation period and when the entire lactation period (LD 1-21) was evaluated. Mean body weights for the 100 mg/kg/day group of F_0 females were significantly but only slightly lower than control over the period of LD 4-17 but were similar to the control group on LD 21. Mean body weights and body weight gains during lactation were unaffected by test substance administration at 5 and 15 mg/kg/day. Significantly lower mean body weights were noted for the 5 mg/kg/day group on LD 11 and 14. However, these differences were considered not to be treatment related, since they were not observed in a dose-responsive manner and did not correlate with other signs of toxicity at this dose level. Consistent with the decreased body weights of the F_0 females in the 100 mg/kg/day during lactation, mean food consumption for this group was significantly lower than the control group from LD 4-7 through LD 17-21 and for the overall lactation period (LD 1-21). Mean food consumption during lactation was basically unaffected by treatment at the 5 and 15 mg/kg/day dose levels. In two measurement periods, LD 7-11 and 11-14 mean food consumption (g/animal/day) for the 5 mg/kg/day group was statistically lower than the control group; due to the absence of a dose-response, these decrements were considered sporadic and not treatment-related. Furthermore, these apparent differences between mean food consumption for the 5 mg/kg/day group and control were no longer significant when mean food consumption was based on body weight (g/kg body weight/day).

The investigators report that no treatment related effects were noted on mean gestation lengths at any dosage level. In point of fact, mean F_0 gestation length in the 100 mg/kg/day dose group (22.2 days) was significantly, but slightly, longer than control (21.8 days); the investigators considered this difference of 0.4 day not to be toxicologically significant comparable to the WIL historical control of 21.9 days. The study author stated, although the difference between the control and 100 mg/kg/day group was significant ($p<0.05$), a difference of 0.4 days is not considered toxicologically significant." This reviewer agrees that there did not appear to be any significant treatment related effects on the process of parturition at any dose level and no treatment related effects on gestation length at the 5 and 15 mg/kg/day dose levels. However, since this difference in mean gestation length is

significant and, although small in magnitude, even a small prolongation in mean gestation length may be consistent with other significant toxicologic effects at the 100 mg/kg/day dose level during gestation (including decreased maternal body weights and body weight gains, decreased food consumption, and various clinical observational findings), the reviewer considers the increased gestation length at the 100 mg/kg/day dose level to be a treatment related effect.

During the dosing period, one F_0 female in the 100 mg/kg/day group was found dead on GD 21. At necropsy, this female had 14 dead fetuses in utero, but no notable macroscopic lesions. All other females survived to the scheduled necropsies. One F_0 female in the control group and 1 F_0 female in the 100 mg/kg/day group failed to deliver and were determined to be nongravid. At terminal necropsies, no treatment related internal findings were observed. Also, no treatment related effects were observed on the number of former implantation sites and the number of unaccounted-for sites.

The maternal LOAEL is 15 mg/kg/day administered orally by gavage daily from gestation day 6 to lactation day 20, primarily based on the various dose-dependent adverse clinical findings for the 15 and 100 mg/kg/day group F_0 females. Post-dosing observations indicative of a deficit in maternal care were clearly associated with the 100 mg/kg/day dose level and similar observations, although occurring to a lesser extent, were noted at the 15 mg/kg/day dose level. Additionally, decrements in body weights and body weight gains, with corresponding reductions in food consumption, were noted only at the 100 mg/kg/day dose level during gestation and lactation. A slight increase in length of gestation was also noted for the 100 mg/kg/day dose group. No significant treatment effects in the F_0 females occurred at the 5 mg/kg/day dose level. The maternal NOAEL for demiditraz is 5 mg/kg/day when administered orally by gavage from gestation day 6 to lactation day 20.

The mean number of pups born per litter and the mean live litter size were slightly, but not significantly, lower in the 100 mg/kg/day group compared with the control group and the mean percentage of males per litter at birth at this dose level was similar to control. Relative to the number of pups born, the mean postnatal survival (% per litter) for the 100 mg/kg/day group was notably lower than the control on PND 4 (pre-culling) but the difference was not statistically significant. The mean number of pups born, live litter size, percentage of males per litter at birth, and immediate postnatal survival were relatively unaffected at the 5 and 15 mg/kg/day.

The only notable general clinical findings for the F_1 offspring over the postnatal period of PND 1 to PND 21 were that more pups were found dead (24 pups in 12 litters) or missing/presumed cannibalized (20 pups in 12 litters) in the 100 mg/kg/day treatment group than in control 11 pups in 9 litters and 3 pups in 3 litters, respectively). Although not statistically significant, this lower pre-weaning pup survival in the 100 mg/kg/day group is considered treatment related. No treatment related effects on pre-weaning pup survival were found for the 5 or 15 mg/kg/day dose groups. The other measures of general physical condition (defined as the occurrence and severity of general clinical findings) of surviving F_1 pups from PND 1 to PND 21 were not remarkably affected by maternal test substance administration. No internal findings attributable to treatment were noted at the necropsies of pups that were found dead at any dose. The investigators concluded that "The general physical condition (defined as the occurrence and severity of clinical findings) of all F_1 pups

in this study was unaffected by maternal test substance administration." The reviewer considers this conclusion, as stated, to be inaccurate, since as noted above there was a treatment related increase in pre-weaning pup deaths in the 100 mg/kg/day dose group and as noted in a subsequent section preweaning body weights/weight gains of F_1 pups were also affected at the 100 mg/kg/day dose level. A more accurate statement would be that, based on the occurrence and severity of the general clinical findings, not including preweaning body weight/weight gain, the general physical condition of surviving F_1 pups was unaffected.

The mean F_1 male and female pup birth weights (PND 1) were significantly lower in the 100 mg/kg/day group compared to the control group and continued to be lower from PND 4 to PND 21, reaching up to 18.5% (males) and 18.3% (females) lower than control. Mean pup body weight gains in the 100 mg/kg/day group males and females were also lower than the control throughout the postnatal period, significantly lower on most measurement intervals. Mean pup body weights and body weight changes in 5 and 15 mg/kg/day group males and females during the pre-weaning period were unaffected by treatment. Pup body weights and weight gains for both sexes in the 5 mg/kg/day dose group were found to be statistically lower than control on several days from PND 14 to 21. However, in view of the absence of a dose-response and the facts that mean body weights of both sexes over these same days for the 5 mg/kg/day group were similar to the mean values in the WIL historical control database and that the control group means were 10% to 11% greater than the historical control means during this time, the body weight/weight gain differences at the 5 mg/kg/day dose level were not considered treatment related.

Body weights for the 100 mg/kg/day group males were significantly lower than control throughout the postweaning period (PND 28-72) and body weights for the females were lower than control from PND 28 to PND 56. Decreased mean body weight gains were also noted for the 100 mg/kg/day group of males, compared with control, generally throughout the entire post-weaning period but females exhibited reduced mean body weight gain only during PND 28-35. No changes in body weight or weight gain were noted in the 5 and 15 mg/kg/day dose groups throughout the postweaning period.

A significant delay in F_1 male sexual maturation (balanopreputial separation) was noted for the 100 mg/kg/day dose group but no such effects were found for the 5 and 15 mg/kg/day groups of males. F_1 female sexual maturation (vaginal patency) was unaffected by treatment at any dose level.

All F_1 animals scheduled for behavioral and neuropathology testing in the control, 5, 15, and 100 mg/kg/day dose groups survived to their scheduled necropsies. No remarkable treatment related general clinical findings were noted during the weekly examinations. In the scheduled detailed clinical observations (functional observational battery) of the F_1 offspring, which were conducted pre- and post-weaning (PND 4, 11, 21, 35, 45, and 60), there were no remarkable treatment related effects on any day of testing in any observational phase (home cage, handling, open field) for either sex at any dose level. There were also no treatment related effects on sensory parameters (i.e., pupillary response) at any dose level. Mean forelimb grip strength scores for the 100 mg/kg/day group of F_1 males and females were significantly lower than control on PND 21 and PND 35 ($p<0.05$ for males and $p<0.01$ for females on both days). Male and female hindlimb grip strength scores at this same dose level were non-significantly lower than controls on both days. On PND 45 and 60, there were no significant treatment effects in male and female forelimb and hindlimb grip strength at the

100 mg/kg/day dose level, although on PND 45 the mean forelimb grip strength for males at this dose level was non-significantly lower than control.
At the 5 and 15 mg/kg/day dose levels, there were no treatment-related effects on forelimb or hindlimb grip strength for either sex, compared to control, at any age of evaluation.

The analyses of motor activity data across sessions PND 13, 17, and 21 focus on determining whether there is an effect of treatment on the ontogenetic pattern of cumulative motor activity across these sessions. There were no statistically significant interactions between treatment and sex. Therefore, subsequent analysis of the treatment by session interaction was conducted on the pooled sexes (males and females combined). The overall treatment by session interactions were significant for both total (p=0.011) and ambulatory (p=0.004) activity, indicating a treatment related change in the developmental pattern of activity across these sessions. Post-hoc pairwise comparisons found that the only statistically significant treatment by combined sessions difference from the control was for the combined sessions at the 5 mg/kg/day dose level for ambulatory activity (p=0.013); although not specifically mentioned in the final report, there were no significant treatment by combined sessions difference on total activity at any dose level and no significant effects on total or ambulatory activity at 15 or 100 mg/kg/day. Based on their inspection of the data, the investigators then concluded that the difference in ontogeny of activity (investigators do not specify whether total or ambulatory) appears to be primarily due to the greater increase in activity from PND 13 to PND 17 in the 5 mg/kg/day dose group compared with the control group (this was based on the significant change in ambulatory activity for sessions at the 5 mg/kg/day dose level). However, this conclusion does not account for the absence of any significant treatment effect for total activity in sessions. To address the significant overall treatment by session interactions that were found for both total and ambulatory activities, the reviewer does not consider it adequate to attribute the difference in ontogeny of activity solely to a greater increase in activity from PND 13 to PND 15 in the 5 mg/kg/day dose group compared with the controls. Rather, based on inspection of the activity data, the reviewer considers it more appropriate at this point to suggest that the difference in ontogeny of activity (considering both total and ambulatory activities) may involve either a greater increase in activity from PND 13 to PND 17 in the 5 mg/kg/day dose group or the lack of a decrease in activity from PND 17 to PND 21 in the 100 mg/kg/day dose group, as compared with control.

To examine the biological significance of this particular aspect of the statistical change in the pattern of ontogeny more closely, the data for females and males for total and ambulatory activity were examined separately in order to compare with historical control data, which is tabulated by sex. The apparent increase in activity for the 5 mg/kg/day dose group on PND 17 (resulting in a greater change from PND 13, compared with control) was found not to be a treatment effect but was due to two outlier animals with unusually high activity counts as compared with the contemporary control and the historical control motor activity data. The investigators concluded that absence of a significant treatment effect on PND 17 at the 5 mg/kg/day dose level indicates that the significant (p=0.011) treatment by session interaction was not a treatment-related effect, but instead caused by outlier pups. The reviewer does not agree with this conclusion as stated, since the absence of a significant effect on either total or ambulatory combined sessions activity for the 5 mg/kg/day group does not necessarily negate the overall treatment by session interaction which was significant for total (p=0.011) and ambulatory activity (p=0.004). Rather, in the opinion of this reviewer, the statement should be that the data merely show that the increased total (non-significant) and ambulatory

(significant) combined sessions activities for the 5 mg/kg/day group were not treatment related effects. Further, as noted below, the significant findings of overall treatment by session interactions for both total activity (p=0.011) and ambulatory activity (p=0.004) may in fact involve between-session differences in activity for the 100 mg/kg/day dose group.

A visual inspection of the data indicates that at the 100 mg/kg/day dose level, but not at the 5 or 15 mg/kg/day dose level, a notable difference in ontogeny of activity (both total and ambulatory) may be particularly associated with a change in activity from PND 17 to PND 21 such that PND 17 activity was either lower than or equivalent to PND 21 activity in the 100 mg/kg/day group compared with control (the expected normal ontogenetic pattern of activity, as is indicated by the control group data, is such that PND 17 activity is notably greater than either PND 13 or PND 21). This type of abnormal pattern of locomotor development may be consistent with a generalized delay in development as evidenced by significant F_1 male and female body weight decreases at the 100 mg/kg/day dose level.

The analyses of activity data within session (PND 13, 17, 21, and 61) focus on determining the effects of treatment on cumulative activity for each session and of treatment on the pattern of habituation within each session. On PND 13, there were no treatment related effects on total and ambulatory motor activity for either the F_1 males or females. Significant decreases in total and ambulatory activities were recorded for F_1 males in the 5 and 15 mg/kg/day dose groups and non-significant decreases for males in the 100 mg/kg/day dose group, when compared with controls. These apparent decreases were determined not to be treatment related effects, since the mean activity scores (total and ambulatory) for 5, 15, and 100 mg/kg/day groups of males were comparable to WIL historical control data, while the concurrent control activities for males on PND 13 were above or near maximum mean values in the historical control data. Also, there was no dose-response relationship in the decreases across the dosed groups of males. On PND 17, 21, and 61, group comparisons with pooled sexes found no significant differences in total or ambulatory activity between the control group and the 5, 15, and 100 mg/kg/day dose groups. Also, there was no treatment related effects on motor activity habituation (i.e., absence of treatment by time interaction) on either test day.

The auditory startle reflex test provides data on sensorimotor function and on habituation which is considered to be a simple form of learning. On PND 20, using pooled sex data there were no significant treatment effects on auditory startle peak amplitude response or on auditory startle habituation (absence of treatment by trial-block interaction) at any dose level. On PND 60, however, analysis of pooled sex data revealed a significant treatment related increase in startle peak amplitude response at the 100 mg/kg/day dose level, but not at the 5 or 15 mg/kg/day dose levels. No treatment effects were found for PND 60 auditory startle habituation at any dose level.

The assessment of learning/memory behaviors was conducted in 3 Phases (Phase 1 - swim ability/motivation; Phase 2 - learning Path A and B; Phase 3 - memory) for both the PND 22 and PND 62 male and female F_1 offspring using the Manual Biel Water Maze. Swimming ability and level of motivation to escape from the water maze of PND 22 and PND 62 male and female F_1 offspring were unaffected by Fo maternal dosing with 5, 15, and 100 mg/kg/day test agent. On PND 22 and PND 62, there were no statistically significant treatment related effects on the mean escape times and mean numbers of errors during the learning (Path A and B) and memory trials for the F_1 males and females in the 5, 15, and 100

mg/kg/day groups, when compared with the control group. On PND 22, however, statistical analysis did reveal significant differences in number of errors in Path B learning for the 5 and 100 mg/kg/day group females, when compared with control; the number of Path B errors for the 15 mg/kg/day group male and female F_1 offspring were similar to control. In general, the significant differences at the 5 and 100 mg/kg/day dose levels involved fewer errors committed by the F_1 females (i.e., the animals learned faster) throughout most of the trials on Path B, compared to the control group. However, the investigators considered these differences in mean numbers of errors between the control and the 5 and 100 mg/kg/day group females not to be treatment related in view of the absence of a dose-response and given that the apparent differences in F_1 female Path B errors, but not males, occurred during PND 22, a period when sex-specific changes are not expected due to the immature state of the animals. The reviewer considers a more compelling rationale for not considering these differences to be treatment related, which was not mentioned in the final report, that is based on the fact that not only were most of the female mean errors (Path B) for the treated groups within 1 standard deviation of the WIL historical control data for PND 22 females but also most of the female errors (Path B) in the 0 mg/kg/day control group were more than one standard deviation higher than the WIL historical control data. Consequently, the differences between the errors (Path B) in the 5 and 100 mg/kg/day females and the unusually higher error scores in the control should be considered false positives and not treatment related effects.

No internal gross findings that could be attributed to any dosage of F_0 maternal treatment were noted at the necropsy of F_1 male and female offspring euthanized on PND 21, offspring euthanized following attainment of sexual developmental landmarks, or offspring not selected for neuropathology and brain weights and euthanized at PND 72.

There were no treatment related macroscopic changes observed in the brain or spinal cord of the F_1 animals (Subset C) selected for neuropathology evaluation on PND 21 or in the brains of F_1 animals (Subset A) selected for neuropathology evaluation on PND 72.

At the 100 mg/kg/day dose level, PND 21 male F_1 offspring were found to have significantly lower brain weights and lower final body weights, with corresponding significantly increased relative brain weights, as compared with controls. The PND 21 females in this group did not have lower brain weight but did have significantly lower final body weight, which resulted in significantly higher relative brain weights, compared to controls. These results indicated that the toxicity at the 100 mg/kg/day dose level appeared to be generalized rather than specifically targeted to the nervous system in that the lower mean brain weights in males were associated with the lower body weights and the higher brain to body weight ratios (relative brain weights) in both male and female offspring reflected the lowered body weights together with the relatively smaller decrease in brain weight or normal brain weight. No effects on brain weights were found for the 5 or 15 mg/kg/day dose groups of F_1 male and female offspring. There were no treatment related effects on brain measurements of length and width at any dose level. On PND 72, the F_1 males in the 100 mg/kg/day dose group had brain weights similar to controls but had significantly lower final body weights with resulting significantly higher relative brain weights, when compared with controls. Note that these PND 72 results were not identified or discussed in the final report. These results also indicated that the toxicity at the 100 mg/kg/day dose level was generalized rather than specifically targeted to the nervous system with the higher relative brain weights reflecting the decreased final body weights together with the normal brain weights. There were no brain

weight or relative brain weight changes on PND 72 in the 100 mg/kg/day females or in either sex at the 5 and 15 mg/kg/day dose levels. There were also no effects of treatment at any dose level on brain measurements of brain length and brain width in the PND 72 male and female offspring.

There were no treatment related microscopic findings in the brains of the PND 21 (Subset C) or PND 72 (Subset A) male and female F_1 offspring at any dose level. Several observations that were reported were generally considered to be spontaneous, apparent in only few animals, similar to findings in the WIL historical control database, common occurrences, incidental findings, or related to some aspect of experimental manipulation (such as over-perfusion) other than administration of the test substance or not dose related and not considered to be treatment related. It should be noted that the reviewer could find no final report Appendix that contained the WIL historical histopathology control data.

There were no treatment related effects on brain morphometry, in the brains of the PND 21 and 72 male and female animals (Subset A and C, respectively) at any dose level. Slight perturbations in several measurements were noted but none were considered biologically relevant.

The F_1 offspring LOAEL is 100 mg/kg/day (the highest dose level used) when administered orally by gavage to the mother daily from gestation day 6 to lactation day 20, based on decreased postnatal survival and pup body weights and body weight gains, increased maximum response to the auditory startle stimulus, decreased total and ambulatory motor activity counts, and decreased grip strength at this dose level. In the absence of any significant treatment effects for the F_1 offspring in either the 5 or 15 mg/kg/day dose groups, the F_1 offspring NOAEL is 15 mg/kg/day when administered orally by gavage to the mother daily from gestation day 6 to lactation day 20.

C. STUDY DEFICIENCIES:

Major Deficiency: The WIL/Kindel Scientific historical control motor activity data (Appendix I in the final report) did not identify the expected normal ontogenetic pattern of motor activity development over the period of PND 13, 17, and 21. This was unexpected particularly since the present study used the same Kindel Scientific equipment that was presumably used to generate the historical data and in the present study the expected normal ontogenetic pattern of motor activity development was produced.

Impact: With reference to this particular study the impact may be considered minor, since historical control motor activity data was typically not the sole rationale for explaining various motor activity aberrancies in the study dataset. However, in terms of the utility of these historical control motor data to aid in the interpretation of findings in subsequent studies, the impact may be considered major, since these historical data do not reliably identify the expected normal ontogenetic pattern of rodent motor activity development over the period of PND 13-21. Furthermore, since the Kindel Scientific locomotor monitoring equipment (and the experimental procedure) that was used in the generation of these historical control data and was also used in the present study in which the expected normal ontogenetic pattern of motor activity development was correctly identified, it will be

important to determine the reason for this discrepancy in order to establish complete confidence in this particular monitoring system and procedure.

Minor Deficiencies:

1) A slight procedural discrepancy was noted regarding the sectioning of brain tissues. The Pathology Report (Appendix F, page 559 of the final report)] stated that "To avoid artifacts in morphometric measurements, a single histotechnologist was responsible for sectioning all brains from a particular age ..". In the description of procedures in the final report itself (page 61) it was stated, as noted above, that "Sectioning was conducted by 2 technicians (1 technician for tissues from males and 1 technician for tissues for females…)". No explanation of this discrepancy was presented.
Suggested Corrective Action: Resolution of this item necessitates clarification by the investigators.

2) Table S35 (page 267 in the final report) which presents the mean data for vaginal patency specifies that N=21 for the 5 mg/kg/day dose group, while all other groups have the expected N=20. This same issue was also noted in the presentation of the Biel water maze data for PND 22 in Table S40 in which all mean values of escape time and number of errors for the 5 mg/kg/day dose group, when pooled sex data were displayed, were reported with N=21. All other PND 22 dose groups and the control group displayed the pooled sex data with the appropriate N=20. No explanations for these discrepancies are presented in the final report.
Suggested Corrective Action: The investigators should review the data files to resolve this item. If the unexpected N values are correct, an explanatory revision to the final report and a re-evaluation of those those data may be needed.

3) The procedures described in the Materials and Methods section of the final report and in the Pathology Report (Appendix F of the final report) state that a maximum of N=10 pup/sex/group were to be used for the histopathology examinations at each age of examination PND 21 and PND 72. As reported in the final report, at the PND 21 (Subset C) examination the 0, 5 and 15 mg/kg/day dose groups each had N=10/sex/group, but the 100 mg/kg/day group had N=11 males and N=13 females. At the PND 72 (Subset A) examination the 5, 15 and 100 mg/kg/day groups each had N=10/sex/group and the 0 mg/kg/day female group had the appropriate N=10 animals, but the 0 mg/kg/day group of males consisted of N=11 animals. No explanation for these discrepancies was provided.
Suggested Corrective Action: The investigators should review the data files to resolve this item. If the unexpected N values are correct, an explanatory revision to the final report and a re-evaluation of those those data may be needed.

4) The reviewer could find no final report Appendix that contained the WIL historical brain histopathology control data. Appendix O, which was identified on pages 103 and 105 of the final report as having the "Historical Control Data" for the results section of "Qualitative Histopathology and Brain Morphometry", contains only historical morphometry data.
Suggested Corrective Action: The correct Appendix with historical brain histopathology control data should be identified and appended to the final report.

5) The description of results in the final report, page 88, mistakenly included PND 4 and 11 as days on which sensory observations (pupil response) were made, although no pupil response data were presented in the final report Table S36 for those days. According the experimental procedures (page 54 of final report), pupil response was one of the parameters not recorded for pups on PND 4 and 11 due to their early stage of development at those times.
Suggested Corrective Action: Appropriate correction to the final report could be made.

6) Details of various test equipment and testing conditions were not provided:

a. For the Biel water maze testing, equipment, including dimensions of the maze and escape platform, water depth, water temperature, and the environmental conditions in the testing room (including lighting, temperature, and humidity) were not provided in the final report.

b. For grip strength, details of the procedure (i.e., equipment, number of test systems, number of animals tested at the same time, dose order of animal testing, manner of handling, number of trials, etc.) and test room environmental conditions (i.e., lighting, temperature, humidity) were not presented in the Procedures section of the final report. Since the final report (page 90) stated that "Positive Control Data" for grip strength were available in Appendix J, attempts were made to obtain some relevant procedural information from that source. Unfortunately, no "Positive Control Data" were provided; the only control data available in Appendix J for grip strength were "Historical Control Data" with no discussion of procedure.

c. For FOB, no information was provided concerning the specific location, or the environmental conditions (e.g., noise level, etc) in which the observations were conducted.
Suggested Corrective Action: Appropriate corrections/additions to the final report could be made.

APPENDIX A

STUDY TYPE: Preliminary Developmental Neurotoxicity Study in rats for dose selection and determination of tissue concentrations of test substance

TEST MATERIAL (PURITY): Demiditraz (PF-03814927) (Purity 100%)

CITATION: Beck, M.J. (2011) An oral (gavage) preliminary developmental neurotoxicity (DNT) study of Demiditraz (PF-03814927) in rats, including exposure assessment. WIL Research Laboratories, LLC, Ashland, Ohio. Laboratory Project ID WIL-344067, Study Initiation March 8, 2011 - Study Completion January 20, 2012. MRID 48766701. Unpublished.

SUMMARY:

The objective of this study (MRID 48766701) was to select dosage levels for a definitive developmental neurotoxicity study and to determine the concentration of the test substance in maternal and neonatal plasma and maternal milk samples.

Four groups (n=10 per group) of mated female Crl:CD(SD) rats (designated F_0 females) were administered the test substance, demiditraz (PF-03814927), in the vehicle (0.5% methylcellulose and 0.1% Tween 80) daily by oral gavage from gestation day 6 through lactation day 12. Dosage levels were 5, 15, 30, and 100 mg/kg/day administered at a dosage volume of 10 mL/kg. A concurrent control group (n = 10 females) received the vehicle on a comparable regimen.

All F_0 females were observed twice daily for morbidity and mortality and general clinical observations were made daily throughout the study (prior to dosing during treatment period). Fo females were also observed daily for signs of toxicity approximately 15-30 minutes after dosing and detailed clinical observations were conducted out of the home cage under blinded conditions on gestation day 16 and lactation day 10 at approximately 20 minutes after dosing. F_0 female body weights and food consumption were recorded at appropriate intervals throughout the study.

Females were allowed to deliver and rear their offspring to lactation day 12. Clinical observations, body weights, and sexes were recorded for the F_1 pups at appropriate intervals.

On PND 11, blood samples (cardiac puncture) were collected from 1 pup/sex/litter at approximately 1, 2, 4, and 6 hours following maternal dose administration. These pups were then euthanized and, after stomach contents were collected, they were discarded without examination. On PND 12, all remaining F_1 pups were euthanized and, after stomach contents were collected from control pups only, they were discarded without examination. On lactation day l2, blood samples (tail vein) were collected from all F_0 females at approximately 30 minutes after dosing and milk samples were collected at approximately 2 hours following dosing. All F_0 females were necropsied on lactation day 12 except one female in the 100 mg/kg/day group that had total litter loss on lactation day 4 and was euthanized at that time.

Clinical findings were noted for F_0 females in a dose-related manner in the 15 (marginal findings), 30, and 100 mg/kg/day groups at 15-30 minutes following dose administration, generally throughout the study. These findings included hypoactivity, a flattened body, sitting with the head held low, shallow respiration, slightly drooping eyelids, and clear material around the mouth in the 15, 30, and 100 mg/kg/day groups; clear or yellow material on various body

surfaces in the 30 and 100 mg/kg/day groups; and hunched posture and rocking, lurching, or swaying while ambulating in the 100 mg/kg/day group. The detailed clinical observations on gestation day 16 and lactation day 10 reported similar findings in the 30 and 100 mg/kg/day group, including a slightly impaired mobility and ataxia in the latter group. No significant clinical findings were noted in the 5 mg/kg/day group of F_0 females.

An initial reduction in mean F_0 body weight gain and a corresponding reduction in mean food consumption were noted in the 100 mg/kg/day group during gestation days 6-9; however, mean body weight gains and food consumption were generally similar to the control group throughout the remainder of gestation and mean body weights in this group were unaffected during gestation. During the lactation treatment period, lower mean body weight gains were noted in the 30 and 100 mg/kg/day groups during lactation days 1-4, with corresponding reduced mean food consumption in the 100 mg/kg/day group throughout the lactation treatment period (lactation days 1-12). As a result, mean body weights in these groups were 5.8% and 7.1% lower, respectively, than the control group on lactation day 4. Mean body weights and body weight gains at 5 and 15 mg/kg/day, and food consumption in the 5, 15, and 30 mg/kg/day groups were similar to the control group throughout the study.

No effects on gestation length or the process of parturition were noted at any dosage level and no significant macroscopic findings were noted at necropsy for F_0 females. In addition, mean numbers of former implantation sites and unaccounted-for sites were similar to the control group at all dosage levels. An increased number of pups found dead and a decrease in mean postnatal survival during PND 1 to 4 (pre-culling) and birth to 4 (pre-selection) were noted in the 100 mg/kg/day group. This was primarily due to 1 total litter loss in this group on PND 4. In addition, lower mean male and female pup birth (PND 1) weights and lower mean male and female pup body weight gains throughout the postnatal period (PND 1-11) resulted in mean male and female pup body weights that were up to 19.6% and 24.4% lower, respectively, than the control group during the postnatal period and a corresponding increased incidence of clinical findings of small stature. Postnatal survival, pup physical condition, and pup body weights were unaffected at dosage levels of 5, 15, and 30 mg/kg/day. The mean numbers of pups born and live litter size at all dosage levels were similar to the control group. No remarkable macroscopic findings were noted at any dosage level at the necropsy of F_1 pups found dead.

The mean concentration of PF-03814927 in dam plasma was 6.60, 27.8, 78.8, and 616 ng/ml in the 5, 15, 30, and 100 mg/kg/day groups, respectively, at approximately 30 minutes post-dosing on lactation day 11. On PND 11, PF-03814927 was quantifiable in neonatal plasma samples collected from pups in the 15, 30, and 100 mg/kg/day groups between 2-6 hours following maternal dose administration. In addition, quantifiable levels of two or more of the metabolites of PF-03814927 were detected in plasma samples from pups in all groups on PND 11. On PND 12, the mean concentration of PF-03814927 in maternal milk samples ranged from 10 - 16% of the mean concentration in dam plasma.

Following administration of demiditraz (PF-03814927) orally by gavage to F_0 female rats once daily from gestation day 6 to lactation day 12, clinical findings for F_0 females were noted at 15 (marginal), 30, and 100 mg/kg/day and lower mean body weights and/or body weight gains were noted for F_0 females at 30 and 100 mg/kg/day with corresponding effects on mean food consumption at 100 mg/kg/day. In addition, increased pup mortality, decreased pup postnatal survival, and decreased pup body weights were noted for F_1 pups at 100 mg/kg/day. Therefore, dosage levels of 5 (no notable effects), 15 (marginal effects), and 100 (significant adverse

effects) mg/kg/day were selected for the definitive developmental neurotoxicity study of demiditraz (PF-03814927) in rats.